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(54) Title: TRP8, A TRANSIENT RECEPTOR POTENTIAL CHANNEL EXPRESSED IN TASTE RECEPTOR CELLS

(57) Abstract: The present invention relates to the discovery, identification and characterization of a transient receptor potential channel, referred to herein as TRP8, which is expressed in taste receptor cells and associated with the perception of bitter and sweet taste. The invention encompasses TRP8 nucleotides, host cell expression systems, TRP8 proteins, fusion proteins, polypeptides and peptides, antibodies to the TRP8 protein, transgenic animals that express a TRP8 transgene, and recombinant "knock-out" animals that do not express TRP8. The invention further relates to methods for identifying modulators of the TRP8-mediated taste response and the use of such modulators to either inhibit or promote the perception of bitterness or sweetness. The modulators of TRP8 activity may be used as flavor enhancers in foods, beverages and pharmaceuticals.

TRP8, A TRANSIENT RECEPTOR POTENTIAL CHANNEL EXPRESSED IN TASTE RECEPTOR CELLS

SPECIFICATION

1. INTRODUCTION

The present invention relates to the discovery, identification and characterization of a transient receptor potential channel, referred to herein as TRP8, which is expressed in taste receptor cells and associated with the perception of bitter and sweet taste. The invention encompasses *TRP8* nucleotides, host cell expression systems, TRP8 proteins, fusion proteins, polypeptides and peptides, antibodies to the TRP8 protein, transgenic animals that express a *TRP8* transgene, and recombinant "knock-out" animals that do not express TRP8. The invention further relates to methods for identifying modulators of the TRP8-mediated taste response and the use of such modulators to either inhibit or promote the perception of bitterness or sweetness. The modulators of TRP8 activity may be used as flavor enhancers in foods, beverages and pharmaceuticals.

2. BACKGROUND OF THE INVENTION

Mammals are generally thought to have five basic categories of taste perception: salt, sour, sweet, bitter and *umami* (monosodium glutamate) (for review, see Lindemann, 1996, *Physiological Reviews*, **76**:719-766; Herness and Gilbertson, 1999, *Annu Rev. Physiol.*, **61**:873:900). The taste signals are sensed by specialized taste receptor cells (TRCs), which are organized into taste buds. Each taste bud comprises between about 50 and 100 individual cells grouped into a cluster that is between 20 and 40 microns in diameter. Nerve fibers enter from the base of the taste bud and synapse onto some of the taste receptor cells. Typically, a single TRC contacts several sensory nerve fibers, and each sensory fiber innervates several TRCs in the same taste bud (Lindemann, *supra*).

TRCs of most, if not all, vertebrate species possess voltage-gated sodium, potassium, and calcium ion channels with properties similar to those of

neurons (Kinnamon, S.C. & Margolskee, R.F., 1996, *Curr. Opin. Neurobiol.* **6**:506-513). Different types of primary tastes appear to utilize different types of transduction mechanisms, and certain types of tastes may employ multiple mechanisms which may reflect varying nutritional requirements amongst species (Kinnamon & Margolskee, *supra*).

Bitter and sweet taste transduction are thought to involve cAMP and IP_3 (Kinnamon & Margolskee, *supra*). The bitter compound denatonium causes calcium ion release from rat TRCs and the rapid elevation of IP_3 levels in rodent taste tissue (*Id.*, citing Bernhardt, SJ. et al., 1996, *J. Physiol. (London)* **490**:325-336 and Akabas, M.H., et al., 1988, *Science* **242**:1047-1050). Since denatonium cannot pass the cell membrane, it has been suggested that it may activate G-protein-coupled receptors, whereby the α and/or $\beta\gamma$ G protein subunits would activate phospholipase C, leading to IP_3 generation and the release of calcium ions (Kinnamon & Margolskee, *supra*).

In recent years, a taste-specific G protein termed "gustducin", which is homologous to the retinal G protein, transducin, has been cloned and characterized (*Id.*, citing McLaughlin, S. et al., 1992, *Nature (London)* **357**:563-569). It is believed that gustducin plays a direct role in both bitter and sweet transduction. For example, gustducin and subunit (α - gustducin) null (knockout) mice had a reduced aversion to bitter compounds. Unexpectedly, the mice also exhibited a preference for sweet compounds suggesting involvement of gustducin in sweet transduction.

Recent biochemical experiments have demonstrated that taste receptor preparations activate transducin and gustducin in response to denatonium and other bitter compounds (Ming et al., 1998, *Proc. Natl. Acad. Sci.* USA **95**:8933-8).

To thoroughly understand the molecular mechanisms underlying taste sensation, it is important to identify each molecular component in the taste signal transduction pathways. The present invention relates to the cloning of an ion channel, TRP8 (transient receptor potential channel 8), that is believed to be involved in taste transduction and may be involved in the changes in intra-cellular calcium ions associated with bitter taste perception.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification and characterization of a transient receptor potential (TRP) channel, referred to hereafter as TRP8, that participates in the taste signal transduction pathway. TRP8 is a channel protein with a high degree of structural similarity to the family of calcium channel proteins known as transient receptor potential channels. As demonstrated by Northern Blot analysis, expression of the *TRP8* transcript is tightly regulated, with the highest level of gene expression found in taste tissue, moderate expression in stomach and small intestine, and very low level expression in uterus and testis. *In situ* hybridization indicated expression of TRP8 in circumvallate and foliate papillae, but not in the surrounding non-gustatory epithelia. Additionally, the general pattern of TRP8 expression was comparable to that of α-gustducin, although the α-gustducin signal was somewhat more intense.

The present invention encompasses *TRP8* nucleotides, host cells expressing such nucleotides and the expression products of such nucleotides. The invention encompasses TRP8 protein, TRP8 fusion proteins, antibodies to the TRP8 channel protein and transgenic animals that express a *TRP8* transgene or recombinant knock-out animals that do not express the TRP8 protein.

Further, the present invention also relates to screening methods that utilize the *TRP8* gene and/or TRP8 gene products as targets for the identification of compounds which modulate, *i.e.*, act as agonists or antagonists, of TRP8 activity and/or expression. Compounds which stimulate taste responses similar to those of bitter tastants can be used as additives to provoke a desired aversive response -- for example to discourage ingestion of compositions containing these compounds by children or animals. Compounds which inhibit the activity of the TRP8 channel may be used to block the perception of bitterness. The inhibitors of TRP8 may be used as flavor enhancers in foods, beverages or pharmaceuticals by decreasing or eliminating the perception of bitter taste.

The invention is based, in part, on the discovery of a channel protein expressed at high levels in taste receptor cells. In taste transduction, bitter compounds

are thought to act via the G-proteins, such as gustducin, which in turn regulate second messenger systems. Co-localization of α-gustducin, γ-gustducin, phospholipase $C\beta_2$ ($PLC\beta_2$) and TRP8 to one subset of taste receptor cells indicates that they may function in the same transduction pathway. It is believed that TRP8 responds to tastant induced inositol triphosphate (IP_3)/diacylglycerol (DAG) generation by flooding the taste cell with extracellular calcium and activating calcium dependent down stream messengers leading to transmitter release into the synapse and activation of afferent gustatory nerves.

3.1. DEFINITIONS

As used herein, italicizing the name of TRP8 shall indicate the TRP8 gene, in contrast to its encoded protein product which is indicated by the name of TRP8 in the absence of italicizing. For example, *"TRP8"* shall mean the TRP8 gene, whereas "TRP8" shall indicate the protein product of the *TRP8* gene.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Nucleotide sequence of the murine *TRP8* cDNA encoding murine TRP8.

Figure 2. Deduced amino acid sequence of the murine TRP8 transient receptor potential channel.

Figure 3A-B. Nucleotide sequence of the human *TRP8* cDNA encoding human TRP8.

Figure 4. Deduced amino acid sequence of the human TRP8 protein transient receptor potential channel.

Figure 5. Amino acid sequence of the murine TRP8 (upper lines); versus human TRP8 (lower lines). Each pair of lines corresponds to a predicted mouse/human exon.

Figure 6A-C. Predicted topography of the TRP8 protein transient receptor potential channel in the membrane.

Figure 7. Distribution of TRP8 mRNA and protein in mouse tissues. (a) Autoradiogram of a northern blot hybridized with a TRP8 cDNA probe. Each lane

contained 25 μg total RNA isolated from the following mouse tissues: circumvallate and foliate papillae-enriched taste tissue (Taste tissue), lingual tissue devoid of taste buds (Non-taste), brain, retina, olfactory epithelium (Olf. Epi.), stomach, small intestine (Small Int.), thymus, heart, lung, spleen, skeletal muscle (Skele. Mus.), liver, kidney, uterus and testis. A 4.5 kb transcript was detected in taste tissue, stomach and small intestine, and to a much lesser extent, in uterus and testis. To control for mRNA quantity the same blot was stripped and reprobed with a β-actin cDNA probe (lower panel). The size in kilobases (kb) of RNA markers is indicated at the right-hand side. (b) Autoradiogram of a western blot probed with an anti-TRP8 antibody. Protein extracts (50 μg) prepared from the murine tissues indicated were electrophoresed, transferred to a nitrocellulose membrane, then the blot incubated with an antibody against the carboxyl-terminal of TRP8. Immunoreactive protein of ~130 kD, the predicted molecular weight of TRP8, was detected in stomach and small intestine; a higher molecular weight species was identified in liver and kidney. Molecular size markers are given in kilodaltons.

Figure 8. TRP8 mRNA is expressed in taste receptor cells. Sections of murine lingual epithelia containing circumvallate and foliate papillae were hybridized with ^{33}P-labeled antisense RNA probes for TRP8 (a,c) and α-gustducin (d), and subjected to autoradiography. Photomicrographs of circumvallate (a) and foliate (b) papillae hybridized to the antisense TRP8 probe demonstrates expression of TRP8 in a subset of TRCs. (d) Shows hybridization of an α-gustducin antisense probe to foliate papillae. Hybridization controls with sense probes showed the absence of non-specific binding of the TRP8 probe (b) or the α-Gustducin probe (e).

Figure 9. Co-localization in TRCs of TRP8 and other signal transduction elements. Immunofluorescence of Gγ13 (a) and TRP8 (b) in the same longitudinal section of mouse taste papillae section: (c) is the overlay of a and b. Immunofluorescence of TRP8 (d) and α-gustducin (e) in the same section: (f) is the overlay of d and e. Immunofluorescence of PLCβ2 (g) and TRP8 (h) in the same section: (i) is the overlay of g and h.

Figure 10. Profiling the pattern of expression of TRP8, α-gustducin, Gβ1, Gβ3, Gγ13 and PLCβ2 in taste tissue and taste cells. Left panel: Southern hybridization to RT-PCR products from murine taste tissue (T) and control non-taste lingual tissue (N). 3'-region probes from TRP8, α-gustducin (Gust), Gβ1, Gβ3, Gγ13, PLCβ2 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were used to probe the blots. Note that TRP8, α-gustducin, Gβ1, Gβ3, Gγ13 and PLCβ2 were all expressed in taste tissue, but not in non-taste tissue. Right panel: Southern hybridization to RT-PCR products from 24 individually amplified taste receptor cells from a transgenic mouse expressing green fluorescent protein (GFP) from the gustducin promoter. 19 cells were GFP-positive (+), 5 cells were GFP-negative (-). Expression of TRP8, α-gustducin, Gβ3, Gγ13 and PLCβ2 was fully coincident. 15 of 19 TRP8-positive cells were also positive for Gβ1. G3PDH served as a positive control to demonstrate successful amplification of products.

Figure 11. TRP8, but not mTrp 1-7, is detected by PCR in taste tissue. PCR amplifications of TRP8 and mTrp 1-7 were performed using non-degenerate primers specific for each Trp family member. Taste cDNA (upper panels) and brain cDNA (lower panels) provided templates for amplification. Amplified material was resolved in a 1.2% agarose gel. Bands of the expected molecular weight were sequenced to verify the identity of the Trp channel amplified. Positive (G3PDH primers) and negative (no primers) controls are shown (right panels).

Figure 12. Heterologous expression of TRP8. *Xenopus* oocytes were injected with 50 ng of TRP8 cRNA (a) or 50 nl of water (b); two days after injection, oocytes were treated with thapsigargin (2 μM), followed by the addition of Ca^{++} (10 mM) or EGTA as indicated (arrows). The traces represent currents induced at negative membrane potentials (command voltage –80 mV). (c) I-V curve for oocytes injected with TRP8 cRNA or water demonstrates a reversal potential, consistent with Ca^{++} activation of the endogenous calcium-activated chloride conductance (ICl_{Ca}). (d) The maximal inward current elicited with external Ca^{++} present in the bathing media for oocytes injected with TRP8 cRNA or water (control).

Figure 13. TRP8 functions as a Ca^{++} channel. *Xenopus* oocytes were injected with 50 ng of TRP8 cRNA (right panels) or 50 nl of water (left panels); two days after injection, oocytes were treated with thapsigargin (2 μM), followed by the addition of Ca^{++} (10 mM).

Figure 14. Potential signal transduction pathways in TRCs utilizing TRP8. Responses to bitter compounds such as denatonium are initiated by binding to one or more gustducin-coupled receptors of the T2R/TBR family. Activation of the gustducin heterotrimer releases its βγ moiety (e.g. Gβ3/Gγ13) which stimulates PLCβ2, resulting in production of IP_3 and DAG. IP_3 binds to its receptors e.g. IP_3R3 and causes the release of Ca^{++} from intracellular stores, triggering activation of TRP8 channels, which ultimately leads to the influx of Ca^{++} through TRP8 channels. DAG may act directly on TRP8 to lead to Ca^{++} influx. Artificial sweeteners may depend on a similar transduction pathway, but with sweet-responsive receptors, e.g., T1R3 coupled to gustducin or other G proteins initiating the signal that leads to the production of IP_3 and DAG and stimulation of TRP8.

5. DETAILED DESCRIPTION OF THE INVENTION

TRP8 is a channel protein that participates in receptor-mediated taste signal transduction and belongs to the family of calcium channel proteins known as transient receptor potential channels (Montell C., 1997, *Mol. Pharmacol.* **52**:755-763) The present invention encompasses *TRP8* nucleotides, TRP8 proteins and peptides, as well as antibodies to the TRP8 protein. The invention also relates to host cells and animals genetically engineered to express the TRP8 channel or to inhibit or "knock-out" expression of the animal's endogenous TRP8.

The invention further provides screening assays designed for the identification of modulators, such as agonists and antagonists, of TRP8 activity. The use of host cells that naturally express TRP8 or genetically engineered host cells and/or animals offers an advantage in that such systems allow the identification of compounds that affect the signal transduced by the TRP8 protein.

Various aspects of the invention are described in greater detail in the subsections below.

5.1. THE *TRP8* GENE

The cDNA sequence and deduced amino acid sequence of murine TRP8 are shown in Figures 1 and 2, respectively. The cDNA and deduced amino acid sequence of human TRP8 are shown in Figures 3 and 4, respectively.

The *TRP8* nucleotide sequences of the invention include: (a) the DNA sequences shown in FIG. 1 or 3 or contained in the cDNA clone pMR24 within E. coli strain XL10 Gold as deposited with the American Type Culture Collection (ATCC Accession No.); (b) nucleotide sequences that encode the amino acid sequence shown in Figure 2 or 4 or the TRP8 amino acid sequence encoded by the cDNA clone pMR24 as deposited with the ATCC; (c) any nucleotide sequence that (i) hybridizes to the nucleotide sequence set forth in (a) or (b) under stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and (ii) encodes a functionally equivalent gene product; and (d) any nucleotide sequence that hybridizes to a DNA sequence that encodes the amino acid sequence shown in Figure 1 or 3, or that is contained in cDNA clone pMR24 as deposited with the ATCC, under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989 supra), yet which still encodes a functionally equivalent TRP8 gene product. Functional equivalents of the TRP8 protein include naturally occurring TRP8 present in species other than mice and humans. The invention also includes degenerate variants of sequences (a) through (d). The invention also includes nucleic acid molecules, that may encode or act as *TRP8* antisense molecules, useful, for example, in *TRP8* gene regulation (for and/or as antisense primers in amplification reactions of *TRP8* gene nucleic acid sequences).

In addition to the *TRP8* nucleotide sequences described above, homologs of the *TRP8* gene present in other species can be identified and readily isolated, without undue experimentation, by molecular biological techniques well known in the art. For example, cDNA libraries, or genomic DNA libraries derived from the organism of interest can be screened by hybridization using the nucleotides described herein as hybridization or amplification probes.

The invention also encompasses nucleotide sequences that encode mutant TRP8s, peptide fragments of the TRP8, truncated TRP8, and TRP8 fusion proteins. These include, but are not limited to nucleotide sequences encoding polypeptides or peptides corresponding to the TM (transmembrane) and/or CD (cytoplasmic) domains of TRP8 or portions of these domains; truncated TRP8s in which one or two of the domains is deleted, e.g., a functional TRP8 lacking all or a portion of the CD region. Certain of these truncated or mutant TRP8 proteins may act as dominant-negative inhibitors of the native TRP8 protein. Nucleotides encoding fusion proteins may include but are not limited to full length TRP8, truncated TRP8 or peptide fragments of TRP8 fused to an unrelated protein or peptide such as an enzyme, fluorescent protein, luminescent protein, *etc.*, which can be used as a marker.

TRP8 nucleotide sequences may be isolated using a variety of different methods known to those skilled in the art. For example, a cDNA library constructed using RNA from a tissue known to express *TRP8* can be screened using a labeled *TRP8* probe. Alternatively, a genomic library may be screened to derive nucleic acid molecules encoding the TRP8 channel protein. Further, *TRP8* nucleic acid sequences may be derived by performing PCR using two oligonucleotide primers designed on the basis of the *TRP8* nucleotide sequences disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express TRP8.

The invention also encompasses (a) DNA vectors that contain any of the foregoing *TRP8* sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing *TRP8* sequences operatively associated with a regulatory element that directs the expression of the TRP8 coding

sequences; (c) genetically engineered host cells that contain any of the foregoing *TRP8* sequences operatively associated with a regulatory element that directs the expression of the TRP8 coding sequences in the host cell; and (d) transgenic mice or other organisms that contain any of the foregoing TRP8 sequences. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression.

5.2. TRP8 PROTEINS AND POLYPEPTIDES

TRP8 protein, polypeptides and peptide fragments, mutated, truncated or deleted forms of the TRP8 and/or TRP8 fusion proteins can be prepared for a variety of uses, including but not limited to the generation of antibodies, the identification of other cellular gene products involved in the regulation of TRP8 mediated taste perception, and the screening for compounds that can be used to modulate taste perception such as bitter blocking agents and taste modifiers.

Figures 2 and 4 show the deduced amino acid sequence of the murine and human TRP8 protein, respectively. The TRP8 amino acid sequences of the invention include the amino acid sequence shown in Figure 2 or Figure 4, or the amino acid sequence encoded by cDNA clone pMR24 as deposited with the ATCC. Further, TRP8s of other species are encompassed by the invention. In fact, any TRP8 protein encoded by the *TRP8* nucleotide sequences described in Section 5.1, above, is within the scope of the invention.

The invention also encompasses proteins that are functionally equivalent to the TRP8 encoded by the nucleotide sequences described in Section 5.1, as judged by any of a number of criteria, including but not limited to the ability of a bitter tastant to trigger the influx of calcium from extracellular calcium stores into a taste receptor cell expressing said protein, leading to transmitter release from the taste receptor cell into the synapse and activation of an afferent nerve. Such functionally equivalent TRP8 proteins include but are not limited to proteins having additions or substitutions of amino acid residues within the amino acid sequence encoded by the

TRP8 nucleotide sequences described, above, in Section 5.1, but which result in a silent change, thus producing a functionally equivalent gene product.

Peptides corresponding to one or more domains of TRP8 (*e.g.*, transmembrane (TM) or cellular domain (CD)), truncated or deleted TRP8s (*e.g.*, TRP8 in which the TM and/or CD is deleted) as well as fusion proteins in which the full length TRP8, a TRP8 peptide or a truncated TRP8 is fused to an unrelated protein are also within the scope of the invention and can be designed on the basis of the *TRP8* nucleotide and TRP8 amino acid sequences disclosed herein. Such fusion proteins include fusions to an enzyme, fluorescent protein, or luminescent protein which provide a marker function.

While the TRP8 polypeptides and peptides can be chemically synthesized (e.g., see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y.), large polypeptides derived from TRP8 and the full length TRP8 itself may be advantageously produced by recombinant DNA technology using techniques well known in the art for expressing a nucleic acid containing *TRP8* gene sequences and/or coding sequences. Such methods can be used to construct expression vectors containing the *TRP8* nucleotide sequences described in Section 5.1 and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. (See, for example, the techniques described in Sambrook et al., 1989, *supra*, and Ausubel et al., 1989, *supra*).

A variety of host-expression vector systems may be utilized to express the *TRP8* nucleotide sequences of the invention. Where the TRP8 peptide or polypeptide is expressed as a soluble derivative (*e.g.*, peptides corresponding to TM and/or CD) and is not secreted, the peptide or polypeptide can be recovered from the host cell. Alternatively, where the TRP8 peptide or polypeptide is secreted the peptide or polypeptides may be recovered from the culture media. However, the expression systems also include engineered host cells that express TRP8 or functional equivalents, anchored in the cell membrane. Purification or enrichment of the TRP8 from such expression systems can be accomplished using appropriate detergents and

lipid micelles and methods well known to those skilled in the art. Such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the TRP8, but to assess biological activity, *i.e.,* in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors containing *TRP8* nucleotide sequences; yeast transformed with recombinant yeast expression vectors containing *TRP8* nucleotide sequences or mammalian cell systems harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells or from mammalian viruses.

Appropriate expression systems can be chosen to ensure that the correct modification, processing and sub-cellular localization of the TRP8 channel protein occurs. To this end, eukaryotic host cells which possess the ability to properly modify and process the TRP8 channel protein are preferred. For long-term, high yield production of recombinant TRP8 channel protein, such as that desired for development of cell lines for screening purposes, stable expression is preferred. Rather than using expression vectors which contain origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements and a selectable marker gene, *i.e., tk, hgprt, dhfr, neo*, and *hygro* gene, to name a few. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in enriched media, and then switched to a selective media. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that modulate the endogenous activity of the TRP8 gene product.

5.3. TRANSGENIC ANIMALS

The TRP8 gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate TRP8 transgenic animals.

Any technique known in the art may be used to introduce the *TRP8* transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, *Proc. Natl. Acad. Sci. USA* **82**:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, *Cell*, **56**:313-321); electroporation of embryos (Lo, 1983, *Mol Cell. Biol.* **3**:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, *Cell* **57**:717-723); etc. For a review of such techniques, see Gordon, 1989, *Transgenic Animals, Intl. Rev. Cytol.* **115**:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the *TRP8* transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, *i.e.*, mosaic animals. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al., (Lasko, M. et al., 1992, *Proc. Natl. Acad. Sci. USA* **89**:6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the *TRP8* transgene be integrated into the chromosomal site of the endogenous *TRP8* gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous *TRP8* gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous *TRP8* gene.

Once transgenic animals have been generated, the expression of the recombinant *TRP8* gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, *in situ*

hybridization analysis, and RT-PCR. Samples of *TRP8* gene-expressing tissue may also be evaluated immunocytochemically using antibodies specific for the *TRP8* transgene product.

5.4. ANTIBODIES TO TRP8 PROTEINS

Antibodies that specifically recognize one or more epitopes of TRP8, or epitopes of conserved variants of TRP8, or peptide fragments of TRP8 are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in conjunction with compound screening schemes, as described, below, in Section 5.5, for the evaluation of the effect of test compounds on expression and/or activity of the TRP8 gene product.

For production of antibodies, various host animals may be immunized by injection with a TRP8 protein, or TRP8 peptide. Such host animals may include but are not limited to rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies comprising heterogeneous populations of antibody molecules, may be derived from the sera of the immunized animals. Monoclonal antibodies may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature*

256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983, *Proc. Natl. Acad. Sci.* USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclasses thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titres of Mabs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used (Morrison et al., 1984, *Proc. Nat'l. Acad. Sci.*, 81:6851-6855; Neuberger et al., 1984, *Nature*, 312: 604-608; Takeda et al. 1985, Nature 314: 452-454). Alternatively, techniques developed for the production of humanized antibodies (U.S. Patent No. 5,585,089) or single chain antibodies (U.S. Patent No. 4,946,778 Bird, 1988, Science 242: 423-426; Huston et al., 1988, *Proc. Nat'l. Acad. Sci USA*, 85: 5879-5883; and Ward et al., 1989, *Nature* 334: 544-546) may be used to produce antibodies that specifically recognize one or more epitopes of TRP8.

5.5. SCREENING ASSAYS FOR DRUGS AND OTHER CHEMICAL COMPOUNDS USEFUL IN REGULATION OF TASTE PERCEPTION

The present invention relates to screening assay systems designed to identify compounds or compositions that modulate TRP8 activity or *TRP8* gene expression, and thus, may be useful for modulation of bitter taste perception.

In accordance with the invention, a cell-based assay system can be used to screen for compounds that modulate the activity of the TRP8 and thereby, modulate the perception of bitterness. To this end, cells that endogenously express *TRP8* can be used to screen for compounds. Alternatively, cell lines, such as 293 cells,

COS cells, CHO cells, fibroblasts, and the like, genetically engineered to express *TRP8* can be used for screening purposes. Preferably, host cells genetically engineered to express a functional TRP8 are those that respond to activation by bitter tastants, such as taste receptor cells. Further, ooyctes or liposomes engineered to express the TRP8 channel protein may be used in assays developed to identify modulators of TRP8 activity.

The present invention provides for methods for identifying a compound that induces the perception of a bitter taste (a "bitterness activator") comprising (i) contacting a cell expressing the TRP8 channel protein with a test compound and measuring the level of TRP8 activation; (ii) in a separate experiment, contacting a cell expressing the TRP8 channel protein with a vehicle control and measuring the level of TRP8 activation where the conditions are essentially the same as in part (i), and then (iii) comparing the level of activation of TRP8 measured in part (i) with the level of activation of TRP8 in part (ii), wherein an increased level of activated TRP8 in the presence of the test compound indicates that the test compound is a TRP8 activator.

The present invention also provides for methods for identifying a compound that inhibits the perception of a bitter taste (a "bitterness inhibitor") comprising (i) contacting a cell expressing the TRP8 channel protein with a test compound in the presence of a bitter tastant and measuring the level of TRP8 activation; (ii) in a separate experiment, contacting a cell expressing the TRP8 channel protein with a bitter tastant and measuring the level of TRP8 activation, where the conditions are essentially the same as in part (i) and then (iii) comparing the level of activation of TRP8 measured in part (i) with the level of activation of TRP8 in part (ii), wherein a decrease level of activation of TRP8 in the presence of the test compound indicates that the test compound is a TRP8 inhibitor.

A "bitter tastant", as defined herein, is a compound or molecular complex that induces, in a subject, the perception of a bitter taste. In particular, a bitter tastant is one which results in the activation of the TRP8 channel protein resulting in an influx of Ca^{+2} into the cell. Examples of bitter tastants include but are

not limited to denatonium benzoate ("denatonium"; also "DEN"), quinine hydrochloride ("quinine"; also "QUI"), strychnine hydrochloride ("strychnine"; also "STR"), nicotine hemisulfate ("nicotine"; also "NIC"), atropine hydrochloride ("atropine"; also "ATR"), sparteine, naringin, caffeic acid("caffeine"; also "CAF"), quinacrine, and epicatechin. *See* Ming et al., 1999, *Proc. Natl. Acad. Sci. U.S.A.* **96**:9903-9908, incorporated by reference herein.

In utilizing such cell systems, the cells expressing the TRP8 channel protein are exposed to a test compound or to vehicle controls (e.g., placebos). After exposure, the cells can be assayed to measure the expression and/or activity of components of the signal transduction pathway of *TRP8*, or the activity of the signal transduction pathway itself can be assayed.

The ability of a test molecule to modulate the activity of *TRP8* may be measured using standard biochemical and physiological techniques. Responses such as activation or suppression of catalytic activity, phosphorylation or dephosphorylation of TRP8 and/or other proteins, activation or modulation of second messenger production, changes in cellular ion levels, association, dissociation or translocation of signaling molecules, or transcription or translation of specific genes may be monitored. In non-limiting embodiments of the invention, changes in intracellular Ca^{2+} levels may be monitored by the fluorescence of indicator dyes such as indo, fura, etc. In addition activation of cyclic nucleotide phosphodiesterase, adenylate cyclase, phospholipases ATPases and Ca^{2+} sensitive release of neurotransmitters may be measured to identify compounds that modulate TRP8 signal transduction. Further, changes in membrane potential resulting from modulation of the TRP8 channel protein can be measured using a voltage clamp or patch recording methods.

For example, after exposure to a test compound, cell lysates can be assayed for increased intracellular levels of Ca^{2+} and activation of calcium dependent down stream messengers such as phosphodiesterase, phospholipases, ATPases or cAMP. The ability of a test compound to increase intracellular levels of Ca^{2+} and activate phosphodiesterase or decrease cAMP levels compared to those levels seen

with cells treated with a vehicle control, indicates that the test compound acts as an agonist (*i.e.*, is a TRP8 activator) and induces signal transduction mediated by the *TRP8* expressed by the host cell. The ability of a test compound to inhibit bitter tastant induced calcium influx and inhibit phosphodiesterase or increase cAMP levels compared to those levels seen with a vehicle control indicates that the test compound acts as an antagonist (*i.e.*, is a TRP8 inhibitor) and inhibits signal transduction mediated by TRP8.

In a specific embodiment of the invention, levels of cAMP can be measured using constructs containing the cAMP responsive element linked to any of a variety of different reporter genes. Such reporter genes may include but are not limited to chloramphenicol acetyltransferase (CAT), luciferase, β-glucuronidase (GUS), growth hormone, or placental alkaline phosphatase (SEAP). Such constructs are introduced into cells expressing TRP8 channel protein thereby providing a recombinant cell useful for screening assays designed to identify modulators of TRP8 activity.

Following exposure of the cells to the test compound, the level of reporter gene expression may be quantitated to determine the test compound's ability to regulate *TRP8* activity. Alkaline phosphatase assays are particularly useful in the practice of the invention as the enzyme is secreted from the cell. Therefore, tissue culture supernatant may be assayed for secreted alkaline phosphatase. In addition, alkaline phosphatase activity may be measured by calorimetric, bioluminescent or chemilumenscent assays such as those described in Bronstein, I. et al. (1994, *Biotechniques* **17**: 172-177). Such assays provide a simple, sensitive easily automatable detection system for pharmaceutical screening.

Additionally, to determine intracellular cAMP concentrations, a scintillation proximity assay (SPA) may be utilized (SPA kit is provided by Amersham Life Sciences, Illinois). The assay utilizes ^{125}I-label cAMP, an anti-cAMP antibody, and a scintillant-incorporated microsphere coated with a secondary antibody. When brought into close proximity to the microsphere through the labeled cAMP-antibody complex, ^{125}I will excite the scintillant to emit light. Unlabeled cAMP

extracted from cells competes with the ^{125}I-labeled cAMP for binding to the antibody and thereby diminishes scintillation. The assay may be performed in 96-well plates to enable high-throughput screening and 96 well-based scintillation counting instruments such as those manufactured by Wallac or Packard may be used for readout.

In yet another embodiment of the invention, levels of intracellular Ca^{2+} can be monitored using Ca^{2+} indication dyes, such as Fluo-3 and Fura-Red using methods such as those described in Komuro and Rakic, 1998, In: The Neuron in Tissue Culture. L.W. Haymes, Ed. Wiley, New York.

Test activators which activate the activity of TRP8, identified by any of the above methods, may be subjected to further testing to confirm their ability to induce a bitterness perception. Test inhibitors which inhibit the activation of TRP8 by bitter tastants, identified by any of the above methods, may then be subjected to further testing to confirm their inhibitory activity. The ability of the test compound to modulate the activity of the TRP8 receptor may be evaluated by behavioral, physiologic, or *in vitro* methods.

For example, a behavioral study may be performed where a test animal may be offered the choice of consuming a composition comprising the putative TRP8 inhibitor and the same composition without the added compound. A preference for the composition comprising a test compound, indicated, for example, by greater consumption, would have a positive correlation with TRP8 inhibitory activity. Additionally, avoidance by a test animal of food containing a putative activator of TRP8 would have a positive correlation with the identification of an bitterness activator.

In addition to cell based assays, non-cell based assay systems may be used to identify compounds that interact with, e.g., bind to TRP8. Such compounds may act as antagonists or agonists of TRP8 activity and may be used to regulate bitter taste perception.

To this end, soluble TRP8 may be recombinantly expressed and utilized in non-cell based assays to identify compounds that bind to TRP8. The recombinantly expressed TRP8 polypeptides or fusion proteins containing one or

more of the domains of TRP8 prepared as described in Section 5.2, infra, can be used in the non-cell based screening assays. For example, peptides corresponding to one or more of the cytoplasmic or transmembrane domains of TRP8, or fusion proteins containing one or more of the cytoplasmic or transmembrane domains of TRP8 can be used in non-cell based assay systems to identify compounds that bind to the cytoplasmic portion of the TRP8; such compounds may be useful to modulate the signal transduction pathway of the TRP8. In non-cell based assays the recombinantly expressed TRP8 may be attached to a solid substrate such as a test tube, microtitre well or a column, by means well known to those in the art (see Ausubel et al., supra). The test compounds are then assayed for their ability to bind to the TRP8.

The TRP8 channel protein may be one which has been fully or partially isolated from other molecules, or which may be present as part of a crude or semi-purified extract. As a non-limiting example, the TRP8 channel protein may be present in a preparation of taste receptor cell membranes. In particular embodiments of the invention, such taste receptor cell membranes may be prepared as set forth in Ming, D. et al., 1998, *Proc. Natl. Sci. U.S.A.* **95**:8933-8938, incorporated by reference herein. Specifically, bovine circumvallate papillae ("taste tissue", containing taste receptor cells), may be hand dissected, frozen in liquid nitrogen, and stored at -80°C prior to use. The collected tissues may then be homogenized with a Polytron homogenizer (three cycles of 20 seconds each at 25,000 RPM) in a buffer containing 10 mM Tris at pH 7.5, 10% vol/vol glycerol, 1 mM EDTA, 1 mM DTT, 10 μg/μl pepstatin A, 10 μg/μl leupeptin, 10 μg/μl aprotinin, and 100 μM 4-(2-amino ethyl) benzenesulfoyl fluoride hydrochloride. After particulate removal by centrifugation at 1,500 x g for 10 minutes, taste membranes may be collected by centrifugation at 45,000 x g for 60 minutes. The pelleted membranes may then be rinsed twice, re-suspended in homogenization buffer lacking protease inhibitors, and further homogenized by 20 passages through a 25 gauge needle. Aliquots may then be either flash frozen or stored on ice until use. As another non-limiting example, the taste receptor may be derived from recombinant clones (*see* Hoon, M.R. et al., 1999 *Cell* **96**, 541-551).

Assays may also be designed to screen for compounds that regulate TRP8 expression at either the transcriptional or translational level. In one embodiment, DNA encoding a reporter molecule can be linked to a regulatory element of the *TRP8* gene and used in appropriate intact cells, cell extracts or lysates to identify compounds that modulate *TRP8* gene expression. Appropriate cells or cell extracts are prepared from any cell type that normally expresses the *TRP8* gene, thereby ensuring that the cell extracts contain the transcription factors required for *in vitro* or *in vivo* transcription. The screen can be used to identify compounds that modulate the expression of the reporter construct. In such screens, the level of reporter gene expression is determined in the presence of the test compound and compared to the level of expression in the absence of the test compound.

To identify compounds that regulate *TRP8* translation, cells or *in vitro* cell lysates containing *TRP8* transcripts may be tested for modulation of *TRP8* mRNA translation. To assay for inhibitors of *TRP8* translation, test compounds are assayed for their ability to modulate the translation of *TRP8* mRNA in *in vitro* translation extracts.

In addition, compounds that regulate TRP8 activity may be identified using animal models. Behavioral, physiological, or biochemical methods may be used to determine whether TRP8 activation has occurred. Behavioral and physiological methods may be practiced *in vivo*. As an example of a behavioral measurement, the tendency of a test animal to voluntarily ingest a composition comprising the bitter tastant, in the presence or absence of test inhibitor, may be measured. If the bitter tastant activates TRP8 in the animal, the animal may be expected to experience a bitter taste, which would discourage it from ingesting more of the composition. If the animal is given a choice of whether to consume a composition containing bitter tastant only (with activated TRP8) or a composition containing bitter tastant together with a bitterness inhibitor (with lower levels of activated TRP8), it would be expected to prefer to consume the composition containing the bitterness inhibitor. Thus, the relative preference demonstrated by the animal inversely correlates with the activation of the TRP8 channel.

Physiological methods include nerve response studies, which may be performed using a nerve operably joined to a taste receptor cell containing tissue, *in vivo* or *in vitro*. Since exposure to bitter tastant which results in TRP8 activation may result in an action potential in taste receptor cells that is then propagated through a peripheral nerve, measuring a nerve response to a bitter tastant is, *inter alia*, an indirect measurement of TRP8 activation. An example of nerve response studies performed using the glossopharyngeal nerve are described in Ninomiya, Y., et al., 1997, *Am. J. Physiol. (London)* **272**:R1002-R1006.

The assays described above can identify compounds which modulate TRP8 activity. For example, compounds that affect TRP8 activity include but are not limited to compounds that bind to the TRP8, and either activate signal transduction (agonists) or block activation (antagonists). Compounds that affect *TRP8* gene activity (by affecting *TRP8* gene expression, including molecules, e.g., proteins or small organic molecules, that affect transcription or interfere with splicing events so that expression of the full length or the truncated form of the TRP8 can be modulated) can also be identified using the screens of the invention. However, it should be noted that the assays described can also identify compounds that modulate TRP8 signal transduction (e.g., compounds which affect downstream signaling events, such as inhibitors or enhancers of G protein activities which participate in transducing the signal activated by tastants binding to their receptor). The identification and use of such compounds which affect signaling events downstream of TRP8 and thus modulate effects of TRP8 on the perception of taste are within the scope of the invention.

The compounds which may be screened in accordance with the invention include, but are not limited to, small organic or inorganic compounds, peptides, antibodies and fragments thereof, and other organic compounds (e.g., peptidomimetics) that bind to TRP8 and either mimic the activity triggered by the natural tastant ligand (i.e., agonists) or inhibit the activity triggered by the natural ligand (i.e., antagonists).

Compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries (see, e.g., Lam, K.S. et al., 1991, Nature 354:82-84; Houghten, R. et al., 1991, Nature 354:84-86); and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; (see, e.g., Songyang, Z. et al., 1993, Cell 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, $F(ab')_2$ and FAb expression library fragments, and epitope binding fragments thereof), and small organic or inorganic molecules.

Other compounds which may be screened in accordance with the invention include but are not limited to small organic molecules that affect the expression of the *TRP8* gene or some other gene involved in the TRP8 signal transduction pathway (e.g., by interacting with the regulatory region or transcription factors involved in gene expression); or such compounds that affect the activity of the TRP8 or the activity of some other intracellular factor involved in the TRP8 signal transduction pathway, such as, for example, a TRP8 associated G-protein.

5.6. COMPOSITIONS CONTAINING MODULATORS OF TRP8 AND THEIR USES

The present invention provides for methods of inhibiting a bitter taste resulting from contacting a taste tissue of a subject with a bitter tastant, comprising administering to the subject an effective amount of a TRP8 inhibitor, such as a TRP8 inhibitor identified by measuring TRP8 activation as set forth in Section 5.5 *supra*. The present invention also provides for methods of inhibiting a bitter taste of a composition, comprising incorporating, in the composition, an effective amount of a TRP8 inhibitor. An "effective amount" of the TRP8 inhibitor is an amount that subjectively decreases the perception of bitter taste and/or that is associated with a detectable decrease in TRP8 activation as measured by one of the above assays.

The present invention further provides for a method of producing the perception of a bitter taste by a subject, comprising administering, to the subject, a composition comprising a compound that activates TRP8 activity such as a bitterness activator identified as set forth in Section 5.5 *supra*. The composition may comprise an amount of activator that is effective in producing a taste recognized as bitter by a subject.

Accordingly, the present invention provides for compositions comprising bitterness activators and bitterness inhibitors. Such compositions include any substances which may come in contact with taste tissue of a subject, including but not limited to foods, beverages, pharmaceuticals, dental products, cosmetics, and wetable glues used for envelopes and stamps.

In one set of embodiments, a bitterness inhibitor is used to counteract the perception of bitterness associated with a co-present bitter tastant. In these embodiments, a composition of the invention comprises a bitter tastant and a bitterness inhibitor, where the bitterness inhibitor is present at a concentration which inhibits bitter taste perception. For example, when the concentration of bitter tastant in the composition and the concentration of bitterness inhibitor in the composition are subjected to an assay as disclosed in Section 5.1 *supra*, the bitterness inhibitor inhibits the activation of TRP8 by the bitter tastant.

The present invention may be used to improve the taste of foods by decreasing or eliminating the aversive effects of bitter tastants. If a bitter tastant is a food preservative, the TRP8 inhibitors of the invention may permit or facilitate its incorporation into foods, thereby improving food safety. For foods administered as nutritional supplements, the incorporation of TRP8 inhibitors of the invention may encourage ingestion, thereby enhancing the effectiveness of these compositions in providing nutrition or calories to a subject.

The TRP8 inhibitors of the invention may be incorporated into medical and/or dental compositions. Certain compositions used in diagnostic procedures have an unpleasant taste, such as contrast materials and local oral anesthetics. The TRP8 inhibitors of the invention may be used to improve the comfort of subjects undergoing

such procedures by improving the taste of compositions. In addition, the TRP8 inhibitors of the invention may be incorporated into pharmaceutical compositions, including tablets and liquids, to improve their flavor and improve patient compliance (particularly where the patient is a child or a non-human animal).

The TRP8 inhibitors of the invention may be comprised in cosmetics to improve their taste features. For example, but not by way of limitation, the TRP8 inhibitors of the invention may be incorporated into face creams and lipsticks. In addition, the TRP8 inhibitors of the invention may be incorporated into compositions that are not traditional foods, beverages, pharmaceuticals, or cosmetics, but which may contact taste membranes. Examples include, but are not limited to, soaps, shampoos, toothpaste, denture adhesive, glue on the surfaces of stamps and envelopes, and toxic compositions used in pest control (*e.g.*, rat or cockroach poison).

6. EXAMPLE: CLONING AND CHARACTERIZATION OF THE *TRP8* GENE

This following subsection describes the isolation and characterization of a transient receptor protein channel referred to as TRP8. The deduced amino acid sequence of TRP8 shows homology with other TRP proteins. Northern Blot analysis indicates high level expression of TRP8 RNA in taste receptor cells.

6.1. MATERIALS AND METHODS

6.1.1 CLONING OF THE TRP8 GENE

Single cell reverse transcription-polymerase chain reaction (RT-PCR) and differential screening were used to clone genes specifically or selectively expressed in the subset of taste receptor cells that are positive for expression of the G protein gustducin. Individual gustducin-positive cells were isolated from mouse circumvallate papillae (Huang et al. 1999 *Nature Neuroscience* **2**: 1055-1062). The mRNAs from individual cells were reverse transcribed into cDNA followed by PCR amplification. Multiple cDNA libraries from single taste receptor cells were constructed by subcloning the amplified cDNAs into bacteriophage vectors. The cDNA libraries were analyzed by differential screening with self-probe (P^{32}-labelled amplified cDNAs from the same cell) and non-self probe (P^{32}-labeled amplified

cDNAs from another taste cell). Hybridization was carried out at 65°C for 20 hours in 0.5 M sodium phosphate buffer (pH 7.3) containing 1% bovine serum albumin and 4% SDS. The membranes were washed twice at 65°C in 0.1% SDS, 0.5xSSC for 20 minutes and one time at 65°C in 0.1% SDS, 0.1xSSC for 15 minutes. The membranes were exposed to X-ray film at -80°C with an intensifying screen for three days. Clones which more strongly hybridize to self probe than to non-self probe were isolated and their inserts sequenced.

Using this clone as a probe (LQSEQ91), a mouse taste tissue cDNA library was screened for full-length clones under the same hybridization conditions as specified above. Sequencing the clones containing the longest inserts produced a full-length clone with greatest similarity to a family of calcium channel proteins known as transient receptor potential (TRP) channels.

25 μg of total RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (P. Chromczynski and N. Sacchi, 1987, *Anal. Biochem.* **162**:156) from the following mouse tissues: taste bud enriched epithelium, non-taste lingual epithelium, brain, retina, olfactory epithelium, stomach, small intestine, liver, spleen, kidney, lung, heart, thymus, uterus, testis and skeletal muscle. The RNAs were electrophoresed on 1.5% agarose gel containing 6.7% formaldehyde, transferred and fixed to a nylon membrane by UV irradiation. The blot was hybridized with a radiolabeled 1.7 kb fragment generated from the 3' -end of mouse TRP 8 cDNA by random priming with Exo(-) Klenow polymerase in the presence of ($\propto$- ^{32}P)-dCTP. The hybridization was carried out in 0.25 M sodium phosphate buffer (pH 7.2) containing 7% SDS at 65°C with agitation for 24 hours. The membrane was washed twice in 20 mM sodium phosphate buffer (pH 7.2) containing 5% SDS at 65°C for 40 minutes and twice in the same buffer containing 1% SDS at 65°C for 40 minutes. The blot was exposed to X-ray film at -80°C with an intensifying screen for 5 days.

A BLAST search of human high throughput DNA sequences and genomic sequences was done using the mouse TRP8 sequence as the query. From this search a BAC clone was identified that contained the entire human TRP8 gene.

The Genscan program was then used to identify the predicted protein-coding exons of the human TRP8 gene. The regions were aligned with the mouse TRP8 cDNA to refine the predicted human TRP8 coding region, leading to deduction of the entire human coding region.

6.1.2. NORTHERN HYBRIDIZATION

Total RNAs were isolated from several mouse tissues using the Trizol reagents, then 25 μg of each RNA was electrophoresed per lane on a 1.5% agarose gel containing 6.7% formaldehyde. The samples were transferred and fixed to a nylon membrane by UV irradiation. The blot was prehybridized at 65°C in 0.25 M sodium phosphate buffer (pH 7.2) containing 7% SDS and 40 μg/ml herring sperm DNA with agitation for 5 hours; hybridization for 20 hours with the ^{32}P-radiolabeled mouse TRP8 probe was carried out in the same solution. The membrane was washed twice at 65°C in 20 mM sodium phosphate buffer (pH 7.2) containing 5% SDS for 40 minutes, twice at 65°C in the same buffer containing 1% SDS for 40 minutes, and once at 70°C in 0.1 x SSC and 0.1% SDS for 30 minutes. The blot was exposed to X-ray film for 3 days at -80°C with dual intensifying screens. The ^{32}P-labeled TRP8 probe was generated by random nonamer priming of a .48 kb cDNA fragment of TRP8 corresponding to the 3'-UTR sequence using Exo(-) Klenow polymerase in the presence of (α-^{32}P)-dCTP.

6.1.3. *IN SITU* HYBRIDIZATION

^{33}P-labeled RNA probes [TRP8 (1.7kb) and α-gustducin (1 kb)] were used for *in situ* hybridization of frozen sections (10 μm) of mouse lingual tissue. Hybridization and washing were as described (Asano-Miyoshi et al., 2000, *Neurosci Lett* 283:61-4). Slides were coated with Kodak NTB-2 nuclear track emulsion and exposed at 4°C for 3 weeks and then developed and fixed.

6.1.4. IMMUNOCYTOCHEMISTRY

Polyclonal antisera against a keyhole limpet hemocyanin-conjugated TRP8 peptide (aa 1028-1049) were raised in rabbits. The PLCβ2 antibody was

obtained from Santa-Cruz Biotechnologies; the anti-α-gustducin and anti-Gγ13 antibodies were as described (Ruiz-Avila et al., 1995, *Nature* 376:80-5; Huang et al., 1999, *Nat Neurosci* 2:1055-62). Ten micron thick frozen sections of murine lingual tissue (previously fixed in 4% paraformaldehyde and cryoprotected in 20% sucrose) were blocked in 3% BSA, 0.3% Triton X-100, 2% goat serum and 0.1% Na Azide in PBS for 1 hour at room temperature and then incubated for 8 hours at 4°C with purified antibody against α-gustducin, or antiserum against TRP8 (1:800). The secondary antibodies were Cy3-conjugated goat-anti-rabbit Ig for TRP8 and fluorescein-conjugated goat-anti-rabbit Ig for PLCβ2, α-gustducin or Gγ13. TRP8 immunoreactivy was blocked by preincubation of the antisera with the immunizing peptides at 20 μM. Preimmune serum did not show any immunoreactivity. Sections were double-immunostained with TRP8 plus one of the following antibodies: anti-PLCβ2, anti-α-gustducin or anti-Gγ13. The sections were incubated sequentially with TRP8 antiserum, anti-rabbit-Ig-Cy3 conjugate, normal anti-rabbit-Ig, anti-PLCβ2 (or anti-α-gustducin or anti-Gγ13) antibody and finally with anti-rabbit-Ig-FITC conjugate with intermittent washes between each step. Control sections that were incubated with all of the above except anti-PLCβ2 (or anti-α-gustducin or anti-Gγ13) antibody did not show any fluorescence in the green channel.

6.1.5. GENE EXPRESSION PROFILING

Single taste receptor cell RT-PCR products (5 μl) were fractionated by size on a 1.6% agarose gel and transferred onto a nylon membrane. The expression patterns of the isolated cells were determined by Southern hybridization with 3'-end cDNA probes for mouse TRP8, α-gustducin, Gβ3, Gγ13, PLCβ2 and G3PDH. Blots were exposed for five hours at -80°C. Total RNAs from a single circumvallate papilla and a similar-sized piece of non-gustatory epithelium were also isolated, reverse transcribed, amplified and analyzed as for the individual cells.

6.1.6. HETEROLOGOUS EXPRESSION

Oocytes were injected with 50 ng of TRP8 cRNA. 48 hours after injection oocytes were incubated in Thapsigargin (2 μM) and X-Rhod-1-AM (the Ca^{++}

sensing dye) for 3 hours at room temperature.

6.2. RESULTS

6.2.1. IDENTIFICATION OF A NOVEL TRP CHANNEL IN TASTE CELLS

Using single cell reverse transcription-polymerase chain reaction, a clone was isolated that was expressed in gustducin-positive cells but absent from gustducin-negative cells. A search of the expressed sequence tag (EST) dbest database found no matches, suggesting that this clone's pattern of expression is highly restricted to tissues not generally found in EST databases, such as taste tissue.

Using this clone as a probe, a mouse taste tissue cDNA library was screened for full-length clones. Sequencing the clones containing the longest inserts produced a full-length clone with the sequence indicated in Figure 1. The deduced amino acid sequence of the cDNA clone is shown in Figure 2.

The isolated cDNA showed the greatest similarity to a family of calcium channel proteins known as transient receptor potential (TRP) channels. The similarity of the isolated clone to this family of proteins indicated that a TRP channel had been identified. Currently seven TRP channels are known to exist, making this clone the eighth member, named by convention *TRP8*. Mouse *TRP8* (TRP8) is most closely related to TRP7 with an identity at the amino acid level of 40%. The predicted topography of the TRP8 channel inserted within the cell membrane is presented in Figure 6A-C.

Based upon homology of the mouse clone with a region of human chromosome 11p15.5 contained in a BAC clone (genebank #AC003693) a human *TRP8* ortholog was identified. The nucleotide sequence of the human TRP8 gene, as well as the deduced amino acid sequence, are depicted in Figures 3A-B and 4, respectively. A comparison of the murine and human TRP 8 proteins is shown in Figure 5. This region of human chromosome 11p15.5 is syntenic with the distal region of mouse chromosome 7. In both cases, TRP8 and h*TRP8* map between genes for Kvlqt1 and TSSC4.

6.2.2. TRP8 IS SELECTIVELY EXPRESSED IN TASTE TISSUE

Although TRP8 was identified during a differential screen of α-gustducin-positive (+) vs. α-gustducin (-) TRCs, it was possible that TRP8 might be more broadly expressed in other taste cells and/or tissues. To determine the tissue distribution of TRP8 mRNA a northern blot with multiple murine tissues was carried out. An TRP8 3'-UTR probe hybridized predominantly to a transcript of 4.5 kb in taste tissue, with no detectable expression in control non-taste tissue. Moderate expression was detected in stomach and small intestine; weak expression was noted in uterus and testis (Figure 7A). This is in contrast to the results of Enklaar et al., (2000, *Genomics* 67:179-87). Using an RT-PCR-generated probe designed to amplify the 3' portion of TRP8's coding region they detected highest expression in liver and low level expression in other peripheral tissues (e.g. heart, brain, kidney and testis). Their RT-PCR probe may have detected by cross-hybridization other TRP8 mRNAs or an alternatively spliced mRNA with a different 3'-end from that present in our 3'-UTR probe. As an independent measure of expression of TRP8, we carried out western blots using an anti-TRP8 antibody (Figure 7B). TRP8 protein of the predicted molecular weight (~130 kDa) was detected in stomach and small intestine; a species of higher than expected molecular weight was identified in liver and kidney and may represent either an TRP8 -related protein or an TRP8 product from an alternatively spliced message.

6.2.3. TRP8 IS EXPRESSED IN PARTICULAR SUBSETS OF TASTE RECEPTOR CELLS

In situ hybridization was used to determine the cellular pattern of expression of TRP8 in mouse TRCs. TRP8 mRNA was observed in TRCs in circumvallate and foliate papillae, but not in the surrounding non-gustatory epithelia (Figure 8). $TRP8^+$ TRCs were present in the majority of the taste buds, although not all TRCs were positive, suggesting restricted expression to a subset of TRCs. The general pattern of TRP8 expression was comparable to that of α-gustducin, although the α-gustducin signal was somewhat more intense (Figure 8D). Controls with sense

probes showed minimal non-specific hybridization to taste tissue with either the TRP8 probe (Figure 8B) or the α-gustducin probe (Figure 8E).

To determine if TRP8 is co-expressed in TRCs with signal transduction elements that might be involved in its activation, we performed single and double immunohistochemistry of TRC-containing tissue sections. TRP8 protein was co-expressed absolutely with Gγ13 (Figure 9ABC) and PLCβ2 (Figure 9GHI), suggesting that these three molecules might be part of a common signal transduction pathway. TRP8 co-expressed largely, but not absolutely, with α-gustducin (Figure 9DEF): a subset of the TRP8$^+$ TRCs were negative for α-gustducin, although all α-gus$^+$ TRCs were positive for TRP8. This pattern is consistent with our observations that α-gus$^+$ TRCs constitute a subset of TRCs that are positive for Gγ13, Gβ1, PLCβ2 and IP_3R3 (Huang et al, 1999,). The slight differences in distribution at the cellular level among the different molecules could be explained by the different topologies that each protein displays: TRP8 is an integral membrane protein, whereas α-gustducin and PLCβ2 are membrane-associated proteins. The expression of human TRP8 (hTRP8) in human fungiform taste buds was also confirmed.

To independently monitor co-expression of TRP8 in TRCs with the above-mentioned signal transduction elements, as well as with Gβ1 and Gβ3, a single cell expression profiling was carried out (Huang et al., 1999, *Nat Neurosci* 2:1055-62)). In this way it was determined that expression of α-gustducin, Gβ1, Gβ3, Gγ13, PLCβ2 and TRP8 was restricted to taste tissue (Figure 10, left panel), and that in this set of 24 TRCs, TRP8 co-expressed absolutely with α-gustducin, Gβ3, Gγ13, PLCβ2 (Figure 10, right panel); expression of TRP8 also overlapped in large part with that of Gβ1 (15 of 19 TRP8$^+$ cells were also Gβ1$^+$). The coincident expression of these various signal transduction molecules with TRP8 could provide the physical opportunity for activation of TRP8 by IP_3 (by activation of IP_3 receptors) or DAG (by direct activation of TRP8) generated by a signaling pathway in which GPCRs coupled to heterotrimeric gustducin (i.e. α-gustducin/β3/γ13) or to other Gα/β1,β3/γ13-containing heterotrimers might release βγ to activate PLCβ2. Consistent with this is the recent identification in TRCs of IP_3 receptor subtype III (IP_3R3), and the

demonstration that IP_3R3 co-localizes in large part with α-gustducin, Gγ13 and PLCβ2 .

6.2.4. OTHER TRP FAMILY MEMBERS ARE NOT DETECTABLY EXPRESSED IN TASTE TISSUE

Native TRP channels are thought to form homo- and hetero-multimers. To identify potential partners for TRP8 in TRCs PCR was used to determine if murine TRP channels 1-6 (TRP 1-6) are expressed in taste tissue (brain tissue provided a positive control). Amplification by the PCR using primer pairs specific for TRP 1-6 identified products of the correct size for all six TRP family members when brain cDNA was used as the template (Figure 11, lower panel); DNA sequencing of these products confirmed amplification of all six TRP family members. TRP8 was not amplified when brain cDNA was the template (Figure 11, lower panel), although it was amplified when taste cDNA provided the template (Figure 11, upper panel) (amplification of TRP8 was confirmed by DNA sequencing). None of the other six TRP family members were amplified when taste tissue cDNA was used as the template (Figure 11, upper panel), suggesting that they are not highly expressed, if at all, in TRCs. In a separate experiment using TRP7 specific primers, TRP7 was detected by PCr in brain cDNA, but not in taste cDNA. Novel TRP channels beyond these seven members might be expressed in TRCs, but at the present time it would appear that TRP8 is the only known TRP channel highly expressed in taste tissue, and as shown above, in TRCs.

6.2.5. EXPRESSED TRP8 ACTS AS A STORE OPERATED CHANNEL

To determine if TRP8 can function as a calcium channel, TRP8 was expressed in *Xenopus* oocytes. The oocytes possess an endogenous calcium-activated chloride conductance (ICl_{Ca}) that may be used to monitor Ca^{++} influx due to activation of store operated Ca^{++} channels belonging to the TRP family. TRP8 RNA obtained by *in vitro* transcription was injected into *Xenopus* oocytes and two electrode voltage clamp recordings were performed two days later. To induce depletion of internal Ca^{++} stores, oocytes were incubated for 2 hours before the recording in 2 μM thapsigargin

(TPN), an irreversible inhibitor of the sarco(endo)plasmic reticulum Ca^{++}-ATPase (SERCA).

Representative recording traces of oocytes injected with TRP8 RNA and treated with TPN demonstrated a robust and distinct inward current elicited by the addition of Ca^{++} to the external bath (Figure 12A). These traces differ dramatically from those of control oocytes injected with water (Figure 12B), indicating that TRP8 encodes a functional Ca^{++} channel whose activation is dependent on the filling status of the internal Ca^{++} stores (compare Figure 12 panels A and B), and whose function relies on the availability of external Ca^{++}. The control oocytes express an endogenous TRP channel (XTrp) (Bobanovic et al., 1999, *Biochem J.* 340:593-9)) that can be activated by TPN treatment (Figure 12B). Analysis of the total inward current (Figure 12D) generated under conditions when Ca^{++} is present in the extracellular medium clearly demonstrated the effect of TRP8 expression. To confirm that TRP8 protein was actually expressed in the oocytes, we carried out a western blot of the membrane proteins from TRP8 RNA-injected oocytes using an anti-TRP8 antibody: a 130 kDa protein of the expected size was detected.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS

1. An isolated nucleic acid molecule comprising a nucleotide sequence that encodes the amino acid sequence shown in Figure 2.

2. The isolated nucleic acid molecule of claim 1 comprising the DNA sequence of FIG. 1.

3. An isolated nucleic acid molecule comprising the DNA sequence of Fig. 3.

4. The isolated nucleic acid molecule of claim 3 comprising a nucleotide sequence that encodes the amino acid sequence shown in Figure 4.

5. An isolated nucleic acid molecule comprising a nucleotide sequence that hybridizes to the nucleotide sequence of Claim 1 or 3 under stringent conditions and encodes a functionally equivalent gene product.

6. An isolated nucleic acid molecule comprising a nucleotide sequence that hybridizes to the nucleic acid of claim 1 or 3 under moderately stringent conditions and encodes a functionally equivalent TRP8 gene product.

7. An isolated nucleic acid molecule that is a *TRP8* antisense molecule.

8. An isolated polypeptide comprising the amino acid sequence of Figure 2.

9. An isolated polypeptide comprising the amino acid sequence of Figure 4.

10. An isolated polypeptide comprising the amino acid sequence encoded by a nucleotide sequence that hybridizes to the nucleotide sequence of Claim 1 or 3 under stringent conditions and encodes a functionally equivalent gene product.

11. An isolated polypeptide comprising the amino acid sequence encoded by a nucleotide sequence that hybridizes to the nucleotide sequence of Claim 1 or 3 under moderately stringent conditions and encodes a functionally equivalent gene product.

12. A purified fragment of a TRP8 protein comprising a domain of the TRP8 protein selected from the group consisting of the transmembrane domain and cytoplasmic domain.

13. A chimeric protein comprising a fragment of a TRP8 protein consisting of at least 6 amino acids fused via a covalent bond to an amino acid sequence of a second protein, in which the second protein is not a TRP8 protein.

14. An antibody which is capable of binding a TRP8 protein.

15. A recombinant cell containing the nucleic acid of claim 5 or 6.

16. A method of producing a TRP8 protein comprising growing a recombinant cell containing the nucleic acid of claim 5 or 6 such that the encoded TRP8 protein is expressed by the cell, and recovering the expressed TRP8 protein.

17. A method for identifying a compound that induces the perception of a bitter taste comprising:

(i) contacting a cell expressing the TRP8 channel protein with a test compound and measuring the level of TRP8 activation;

(ii) in a separate experiment, contacting a cell expressing the TRP8 channel protein with a vehicle control and measuring the level of TRP8 activation where the conditions are essentially the same as in part (i); and

(iii) comparing the level of activation of TRP8 measured in part (i) with the level of activation of TRP8 in part (ii),

wherein an increased level of activated TRP8 in the presence of the test compound indicates that the test compound is a TRP8 inducer.

18. A method for identifying a compound that inhibits the perception of a bitter taste and/or promotes the perception of a sweet taste comprising:

(i) contacting a cell expressing the TRP8 channel protein with a test compound in the presence of a bitter tastant and measuring the level of TRP8 activation;

(ii) in a separate experiment, contacting a cell expressing the TRP8 channel protein with a bitter tastant and measuring the level of TRP8 activation, where the conditions are essentially the same as in part (i); and

(iii) comparing the level of activation of TRP8 measured in part (i) with the level of activation of TRP8 in part (ii),

wherein a decrease level of activation of TRP8 in the presence of the test compound indicates that the test compound is a TRP8 inhibitor.

19. A method for identifying an inhibitor of bitter taste *in vivo* comprising:

(i) offering a test animal the choice of consuming either (a) a composition comprising a bitter tastant or (b) the composition comprising the bitter tastant as well as a test inhibitor; and

(ii) comparing the amount of consumption of the composition according to (a) or (b),

wherein greater consumption of the composition according to (b) has a positive correlation with an ability of the test inhibitor to inhibit the perception of bitter taste associated with the tastant.

20. A method for identifying an activator of bitter taste *in vivo* comprising:

(i) offering a test animal the choice of consuming either (a) a control composition or (b) the composition comprising a test activator; and

(ii) comparing the amount of consumption of the composition according to (a) or (b),

wherein greater consumption of the composition according to (a) has a positive correlation with an ability of the test activator to activate the perception of bitter taste.

21. A method of inhibiting a bitter taste resulting from contacting a taste tissue of a subject with a bitter tastant, comprising administering to the subject an effective amount of a bitterness inhibitor.

22. A method of producing the perception of a sweet taste by a subject, comprising administering, to the subject, a composition comprising a compound that acts as a bitterness inhibitor in addition to eliciting a sweet taste.

23. A method of producing the perception of a bitter taste by a subject, comprising administering, to the subject, a composition comprising a compound that acts as a bitterness activator.

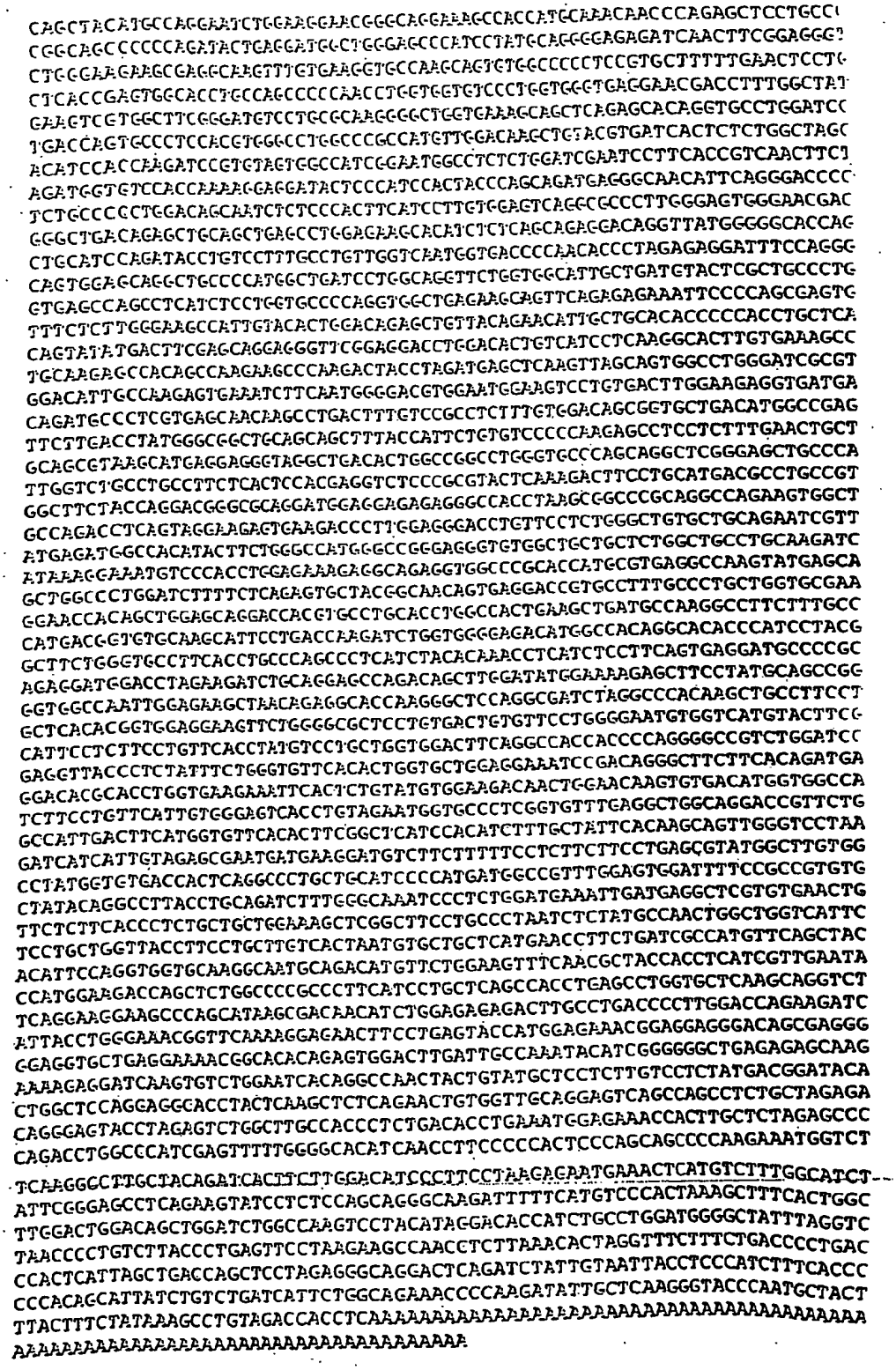


Figure 1

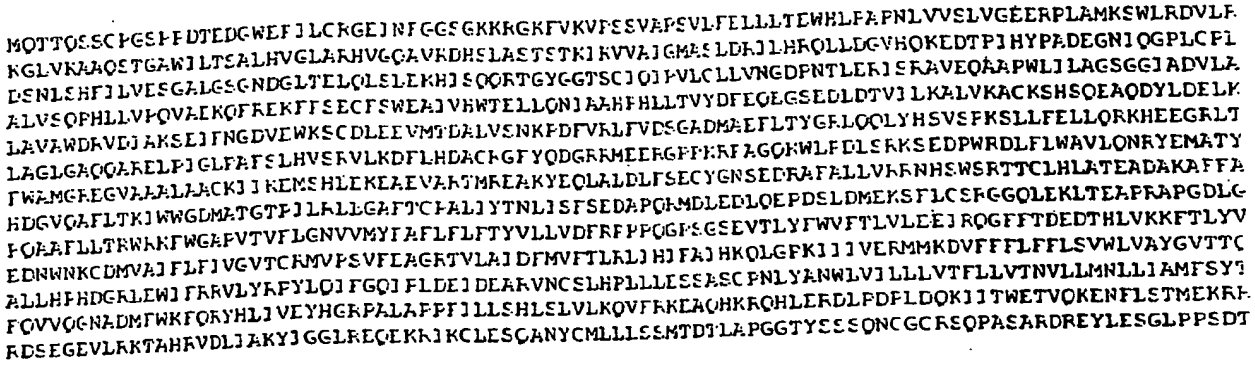


Figure 2

Human TRPt nucleotide sequence
SEQ New: 3498 bp;
Composition: 634 A; 1089 C; 1143 G; 632 T; 0 OTHER
Percentage: 18% A; 31% C; 33% G; 18% T; 0%OTHER

Molecular Weight (kDa): ssDNA: 1081.34 dsDNA: 2157.3
ORIGIN

```
1       ATGCAGGATG TCCAAGGCCC CCGTCCCCGA AGCCCCGGGG ATGCTGAAGA CCGGCGGGAC
61      CTGGGCTTGC ACAGGGGCGA GGTCAACTTT GGAGGGTCTG GGAAGAAGCG AGGCAAGTTT
121     GTACGGGTGC CGAGCGGAGT GGCCCCGTCT GTGCTCTTTG ACCTGCTGCT TGCTGAGTGG
181     CACCTGCCGG CCCCCAACCT GGTGGTGTCC CTGGTGGGTG AGGAGCAGCC TTTCGCCATC
241     AAGTCCTGGC TGCGGGATGT GCTGCGCAAG GGGCTGGTGA AGGCGGCTCA GAGCACAGGA
301     GCCTGGATCC TGACCAGTGC CCTCCGCGTG GGCCTGGCCA GGCATGTCGG GCAGGCCGTG
361     CGCGACCACT CGCTGGCCAG CACGTCCACC AAGGTCCGTG TGGTTGCTGT CGGCATGGCC
421     TCGCTGGGCC GCGTCCTGCA CCGCCGCATT CTGGAGGAGG CCCAGGAGGA TTTTCCTGTC
481     CACTACCCTG AGGATGACGG CGGCAGCCAG GGCCCCCTCT GTTCACTGGA CAGCAACCTC
541     TCCCACTTCA TCCTGGTGGA GCCAGGCCCC CCGGGGAAGG GCGATGGGCT GACGGAGCTG
601     CGGCTGAGGC TGGAGAAGCA CATCTCGGAG CAGAGGGCGG GCTACGGGGG CACTGGCAGC
661     ATCGAGATCC CTGTCCTCTG CTTGCTGGTC AATGGTGATC CCAACACCTT GGAGAGGATC
721     TCCAGGGCCG TGGAGCAGGC TGCCCCGTGG CTGATCCTGG TAGGCTCGGG GGGCATCGCC
781     GATGTGCTTG CTGCCCTAGT GAACCAGCCC CACCTCCTGG TGCCCAAGGT GGCCGAGAAC
841     CAGTTTAAGG AGAAGTTCCC CAGCAAGCAT TTCTCTTGGG AGGACATCGT GCGCTGGACC
901     AAGCTGCTGC AGAACATCAC CTCACACCAG CACCTGCTCA CCGTGTATGA CTTCGAGCAG
961     GAGGGCTCCG AGGAGCTGGA CACGGTCATC CTGAAGGCGC TGGTGAAAGC CTGCAAGAGC
1021    CACAGCCAGG AGCCTCAGGA CTATCTGGAT GAGCTCAAGC TGGCCGTGGC CTGGGACCGC
1081    GTGGACATCG CCAAGAGTGA GATCTTCAAT GGGGACGTGG AGTGGAAGTC CTGTGACCTG
1141    GAGGAGGTGA TGGTGGACGC CCTGGTCAGC AACAAGCCCG AGTTTGTGCG CCTCTTTGTG
1201    GACAACGGCG CAGACGTGGC CGACTTCCTG ACGTATGGGC GGCTGCAGGA GCTCTACCGC
1261    TCCGTGTCAC GCAAGAGCCT GCTCTTCGAC CTGCTGCAGC GGAAGCAGGA GGAGGCCCGC
1321    CTGACGCTGG CCGGCCTGGG CACCCAGCAG GCCCGGGAGC CACCCGCGGG GCCACCGGCC
1381    TTCTCCCTGC ACGAGGTCTC CCGCGTACTC AAGGACTTCC TGCAGGACGC CTGCCGAGGC
1441    TTCTACCAGG ACGGCCGGCC AGGGGACCGC AGGAGGGCGG AGAAGGGCCC GGCCAAGCGC
1501    CCCACGGGCC AGAAGTGGCT GCTGGACCTG AACCAGAAGA GCGAGAACCC CTGGCGGGAC
1561    CTGTTCCTGT GGGCCGTGCT GCAGAACCGC CACGAGATGG CCACCTACTT CTGGGCCATC
1621    GGCCAGGAAG GTGTGGCAGC CGCACTGGCC GCCTGCAAAA TCCTCAAAGA GATGTCGCAC
1681    CTGGAGACGG AGGCCGAGGC GGCCCGAGCC ACGCGCGAGG CGAAATACGA GCGGCTGGCC
1741    CTTGACCTCT TCTCCGAGTG CTACAGCAAC AGTGAGGCCC GCGCCTTCGC CCTGCTGGTG
1801    CGCCGGAACC GCTGCTGGAG CAAGACCACC TGCCTGCACC TGGCCACCGA GGCTGACGCC
1861    AAGGCCTTCT TTGCCCACGA CGGCGTTCAG GCCTTCCTGA CCAGGATCTG GTGGGGGGAC
1921    ATGGCCGCAG GCACGCCCAT CCTGCGGCTG CTAGGAGCCT TCCTCTGCCC CGCCCTCGTC
1981    TATACCAACC TCATCACCTT CAGTGAGGAA GCTCCCCTGA GGACAGGCCT GGAGGACCTG
2041    CAGGACCTGG ACAGCCTGGA CACGGAGAAG AGCCCGCTGT ATGGCCTGCA GAGCCGGGTG
2101    GAGGAGCTGG TGGAGGCGCC GAGGGCTCAG GGTGACCGAG GCCCACGTGC TGTCTTCCTG
2161    CTCACACGCT GGCGGAAATT CTGGGGCGCT CCCGTGACTG TGTTCCTGGG GAACGTGGTC
2221    ATGTACTTCG CCTTCCTCTT CCTGTTCACC TACGTCCTGC TGGTGGACTT CAGGCCGCCC
2281    CCCCAGGGCC CCTCAGGGCC CGAGGTCACC CTCTACTTCT GGGTCTTTAC GCTGGTGCTG
2341    GAGGAAATCC GGCAGGGCTT CTTCACAGAC GAGGACACAC ACCTGGTGAA GAAGTTCACA
2401    CTGTATGTGG GGGACAACTG GAACAAGTGT GACATGGTGG CCATCTTCCT GTTCATCGTG
2461    GGTGTCACCT GCAGGATGCT GCCGTCGGCG TTTGAGGCTG GCCGCACGGT CCTCGCCATG
2521    GACTTCATGG TGTTCAGGCT GCGGCTGATC CATATCTTTG CCATACACAA GCAGCTGGGC
2581    CCCAAGATCA TCGTGGTAGA GCGCATGATG AAGGACGTCT TCTTCTTCCT CTTCTTTCTG
2641    AGCGTGTGGC TCGTGGCCTA CGGTGTCACC ACCCAGGCGC TGCTGCACCC CCATGACGGC
2701    CGCCTGGAGT GGATCTTCCG CCGGGTGCTC TACCGGCCCT ACCTGCAGAT CTTCGGCCAG
2761    ATCCCACTGG ACGAGATTGA TGAAGCCCGT GTGAACTGCT CCACCCACCC ACTGCTGCTG
2821    GAGGACTCAC CATCCTGCCC CAGCCTCTAT GCCAACTGGC TGGTCATCCT CCTGCTGGTC
2881    ACCTTCCTGT TGGTGACGAA TGTGCTGCTC ATGAACCTGC TCATCGCCAT GTTCAGCTAC
2941    ACGTTCCAGG TGGTGCAGGG CAACGCAGAC ATGTTCTGGA AGTTCCAGCG CTACAACCTG
```

Figure 3A

```
301   ATTGTGGAGT ACCACGAGCG CCCCGCCCTG GCCCCGCCCT TCATCCTGCT CAGCCACCTG
361   AGCCTGACGC TCCGCCGGGT CTTCAAGAAG GAGGCTGAGC ACAAGCGGGA GCACCTGGAG
421   AGAGACCTGC CAGACCCCCT GGACCAGAAG GTCGTCACCT GGGAGACAGT CCAGAAGGAG
481   AACTTCCTGA GCAAGATGGA GAAGCGGAGG AGGGACAGCG AGGGGGAGGT GCTGCGGAAA
541   ACCGCCCACA GAGTGGACTT CATTGCCAAG TACCTCCTGG GGCTGAGAGA GCAAGAAAAG
601   CGCATCAAGT GTCTGGAGTC ACAGATCAAC TACTGCTCGG TGCTCGTGTC CTCCGTGGCT
661   GACGTGCTGG CCCAGGGTGG CGGCCCCCGG AGCTCTCAGC ACTGTGGCGA GGGAAGCCAC
721   CTGGTGGCTG CTGACCACAG AGGTGGTTTA GATGGCTGGG AACAACCCGG GGCTGGCCAC
781   CCTCCCTCGG ACACATGA
```

Figure 3B

Human TRPt protein coding sequence
Translation of Htrpbcoding(1-3498
Universal code
Total amino acid number: 1165, MW=131286
Max ORF: 1-3498, 1165 AA, MW=131286

ORIGIN

```
          MQDVQGFRPG SPGDAEDRRE LGLHRGEVNF GGSGKKRGKF VRVPSGVAPS
   51     VLFDLILAEW HLPAPNLVVS LVGEEQHFAM KSWLRDVLRK GLVKAAQSTG
  101     AWILTSALRV GLARHVGQAV RDHCLASTST KVRVVAVGMA SLGRVLHRRI
  151     LEEAQEDFPV HYPEDDGGSQ GPLCSLDSNL SHFILVEPGP PGKGDGLTEL
  201     RLRLEKHISE QRAGYGGTGS IEIFVLCLLV NGDFNTLERI SRAVEQAAPW
  251     LILVGSGGIA DVLAALVNQF HLLVPKVAEK QFKEKFPSKH FSWEDIVRWI
  301     KLLQNITSHQ HLLTVYDFEQ EGSEELDTVI LKALVKACKS HSQEPQDYLI
  351     ELKLAVAWDR VDIAKSEIFN GDVEWKSCDL EEVMVDALVS NKPEFVRLFV
  401     DNGADVADFL TYGRLQELYR SVSRKSLLFD LLQRKQEEAR LTLAGLGTQQ
  451     AREFFAGFPA FSLHEVSRVL KDFLQDACRG FYQDGRPGDR RRAEKGPAKF
  501     FTGQKWLLDL NQKSENFWRD LFLWAVLQNF HEMATYFWAM GQEGVAAALA
  551     ACKILKEMSH LETEAEAARA TREAKYERLA LDLFSECYSN SEARAFALLV
  601     RENRCWSKTT CLHLATEADA KAFFAHDGVQ AFLTRIWWGD MAAGTPILRL
  651     LGAFLCFALV YTNLITFSEE AFLRTGLEDL QDLDSLDTEK SPLYGLQSRV
  701     EELVEAPRAQ GDRGPRAVFL LTRWRKFWGA PVTVFLGNVV MYFAFLFLFI
  751     YVLLVDFRPP PQGPSGPEVT LYFWVFTLVL EEIRQGFFTD EDTHLVKKFT
  801     LYVGDNWNKC DMVAIFLFIV GVTCRMLPSA FEAGRTVLAM DFMVFTLRLI
  851     HIFAIHKQLG PKIIVVERMM KDVFFFLFFL SVWLVAYGVT TQALLHPHDG
  901     RLEWIFRRVL YRPYLQIFGQ IPLDEIDEAR VNCSTHPLLL EDSPSCPSLY
  951     ANWLVILLLV TFLLVTNVLL MNLLIAMFSY TFQVVQGNAD MFWKFQRYNL
 1001     IVEYHERPAL APPFILLSHL SLTLRRVFKK EAEHKREHLE RDLPDPLDQP
 1051     VVTWETVQKE NFLSKMEKRR RDSEGEVLRK TAHRVDFIAK YLGGLREQEK
 1101     RIKCLESQIN YCSVLVSSVA DVLAQGGGPR SSQHCGEGSQ LVAADHRGGI
 1151     DGWEQPGAGQ PPSDT*
```

Figure 4

Figure 5 - Comparison of the amino acid sequences of mouse and human TRPM

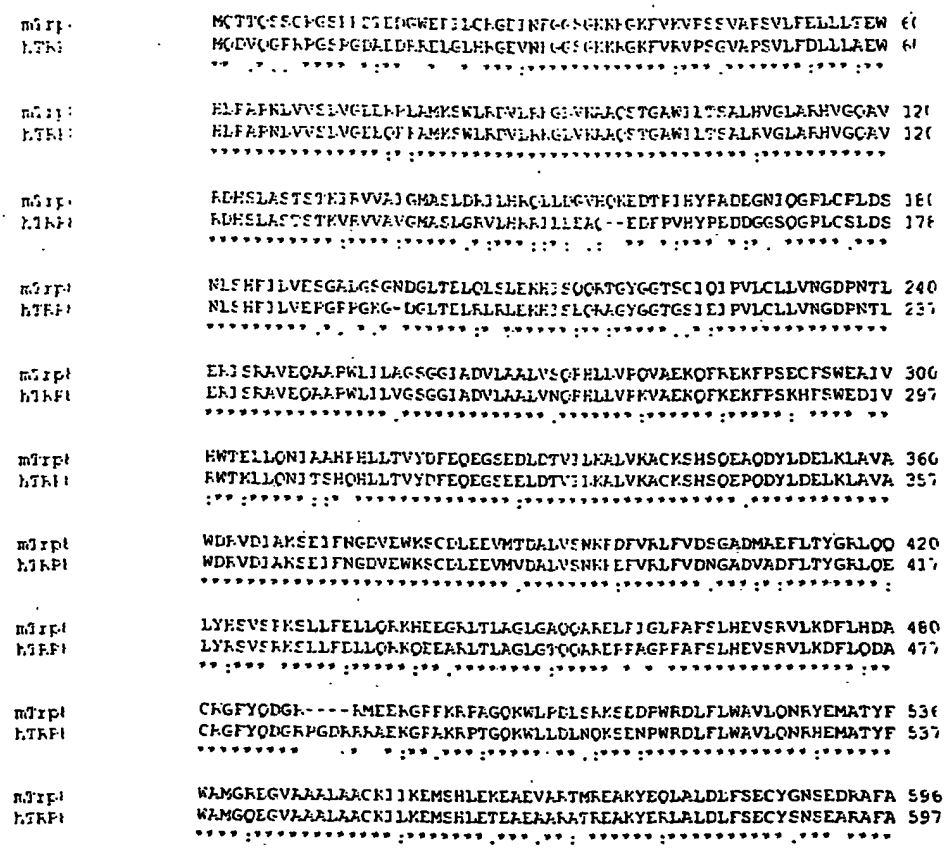


Figure 5

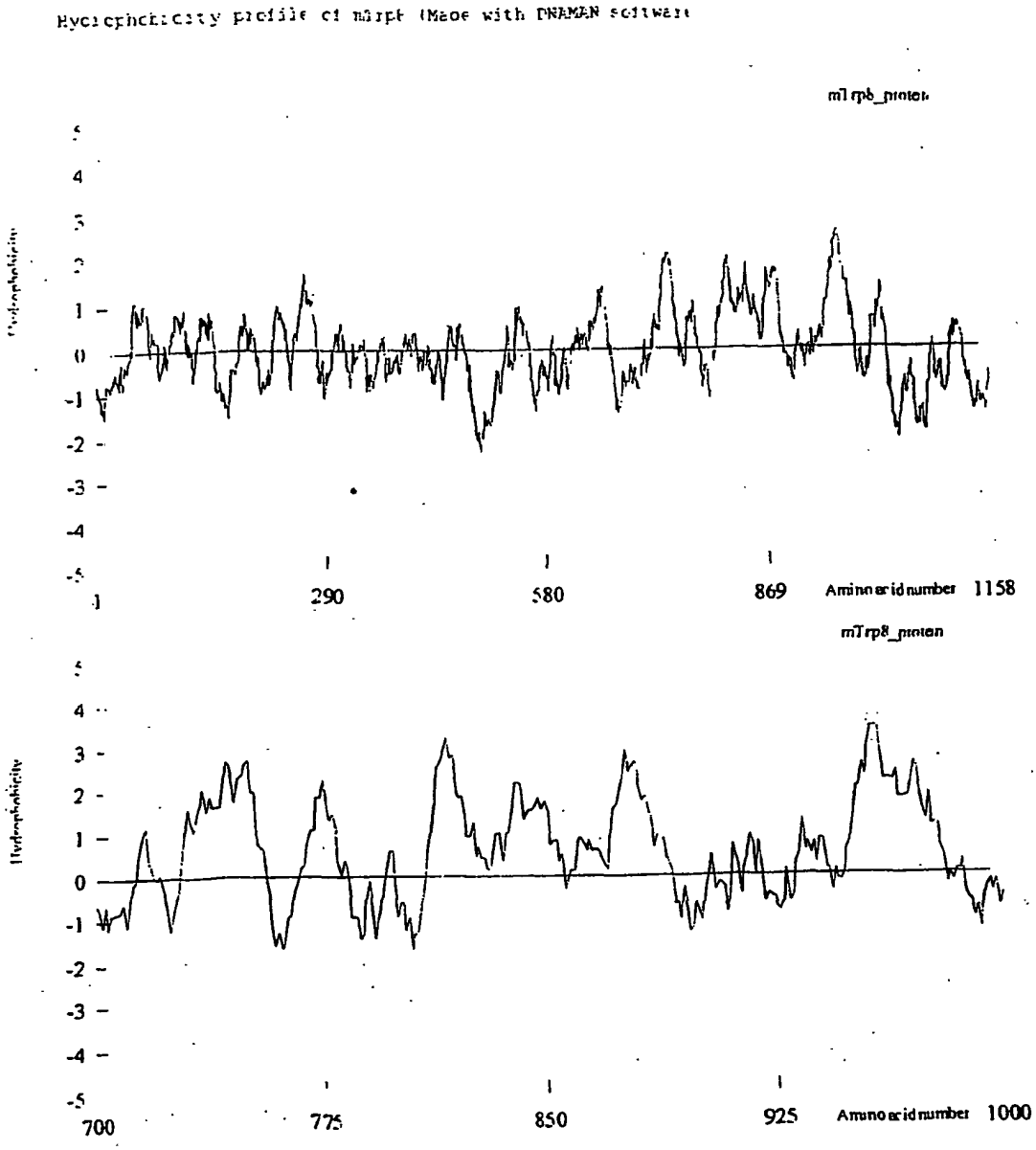
Classification and Secondary Structure Prediction of Membrane Proteins

http://azusa.proteome.bio.tuat.ac.jp/sosui/:

Orientation of the N-terminus of	mTrp8:	IN		
Number of transmembrane helices of	mTrp8:	6		
Position of transmembrane helices of	mTrp8:	helix	begin	end
		1	732	754
		2	769	791
		3	807	829
		4	839	861
		5	870	891
		6	951	973

Orientation of the N-terminus of	hTrp8:	IN		
Number of transmembrane helices of	hTrp8:	6		
Position of transmembrane helices of	hTrp8:	helix	begin	end
		1	733	755
		2	770	792
		3	807	829
		4	841	863
		5	871	893
		6	951	973

Figure 6A


Hydrophobicity profile of mTrp8 made with DNAMAN software
mTrp8_protein
Hydrophobicity
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4
3
2
1
0
-1
-2
-3
-4
-5
1
290
580
869
Amino acid number
1158
mTrp8_protein
Hydrophobicity
5
4
3
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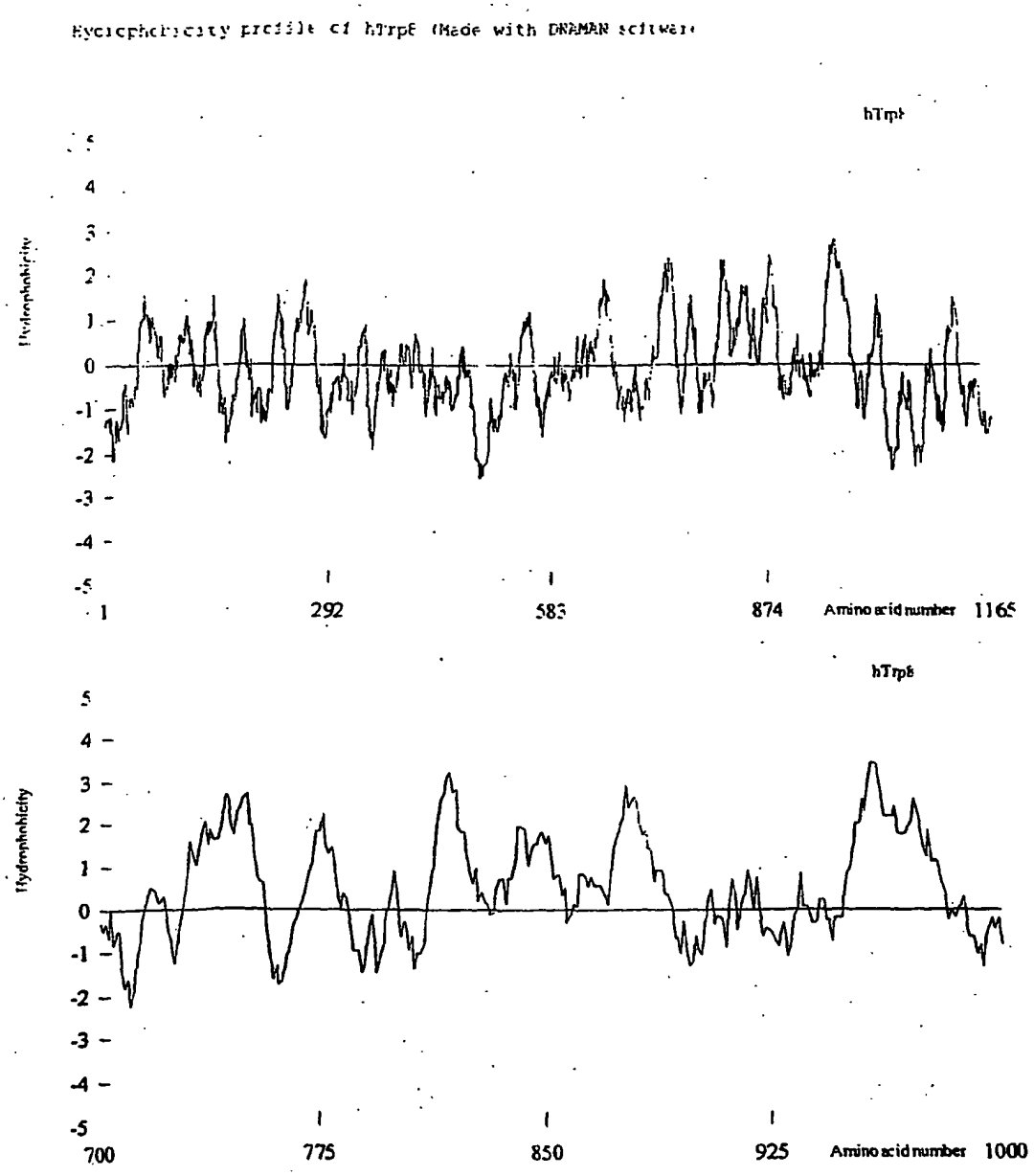
775
850
925
Amino acid number
1000

Figure 6B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hTrpδ
Hydrophobicity
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4
3
2
1
0
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-4
-5
1
292
583
874
Amino acid number
1165
hTrpδ
Hydrophobicity
5
4
3
2
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-5
700
775
850
925
Amino acid number
1000

Figure 6C

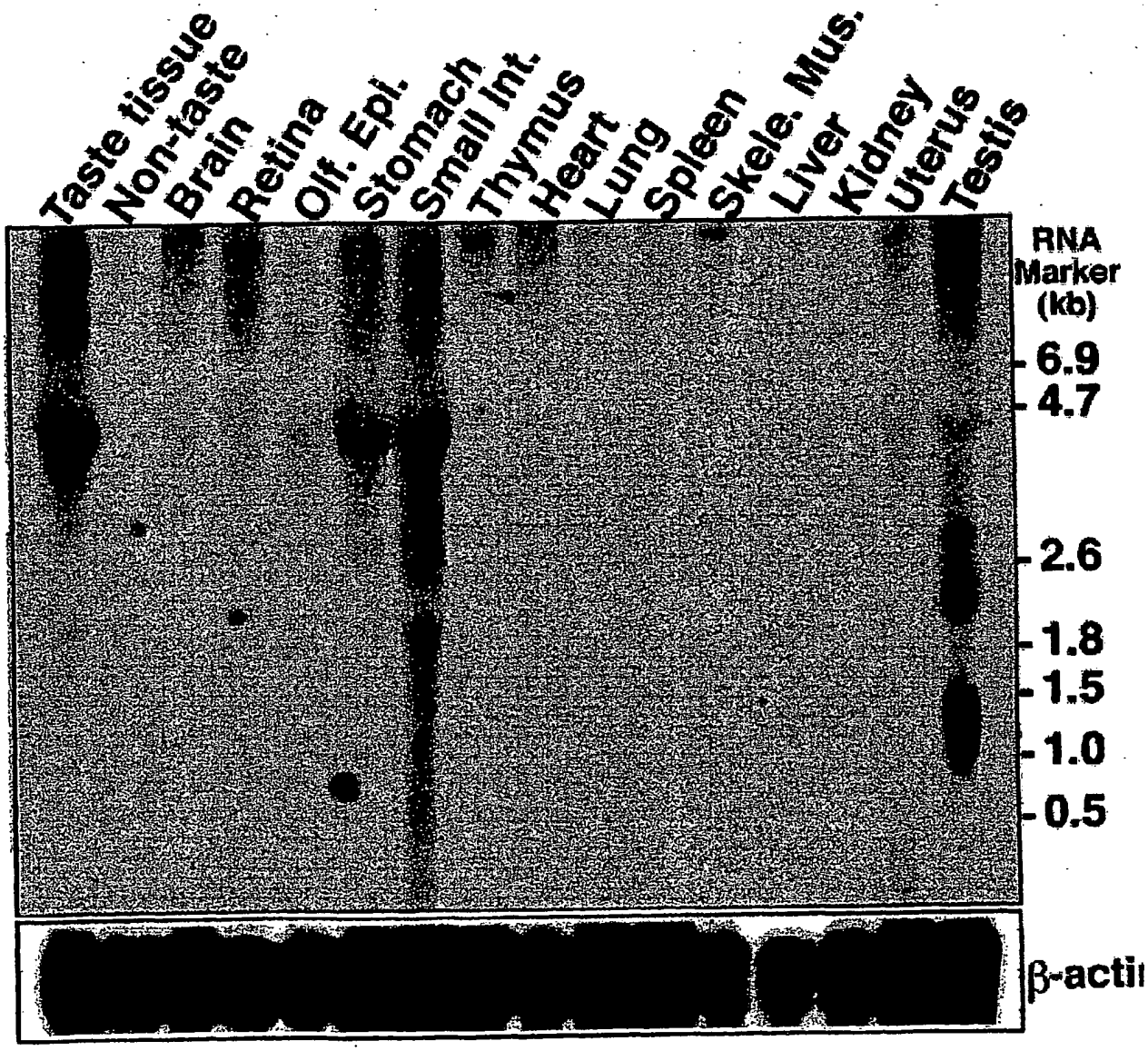
Taste tissue
Non-taste
Brain
Retina
Olf. Epi.
Stomach
Small Int.
Thymus
Heart
Lung
Spleen
Skele. Mus.
Liver
Kidney
Uterus
Testis
RNA Marker (kb)
6.9
4.7
2.6
1.8
1.5
1.0
0.5
β-actin

Figure 7

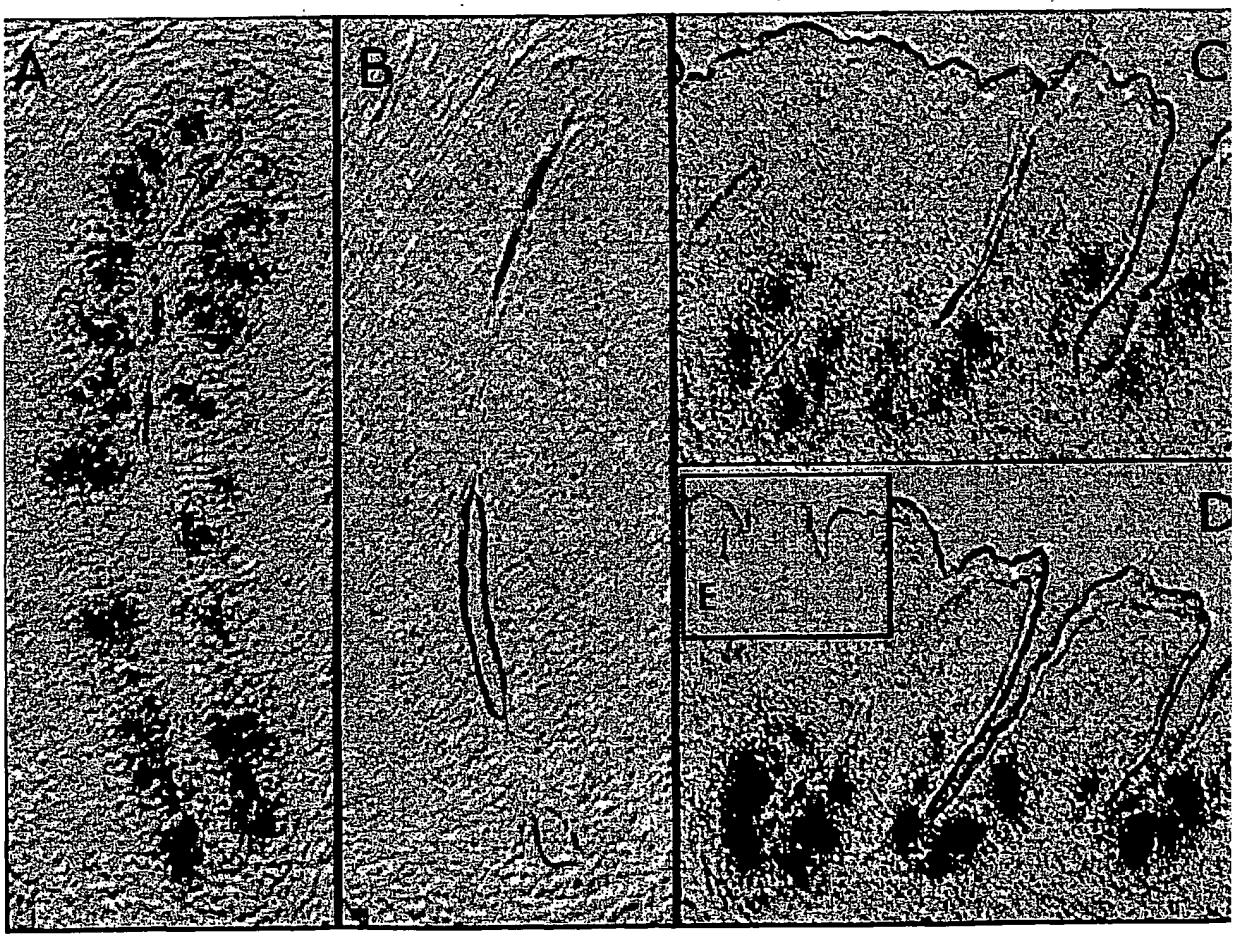

Figure 8

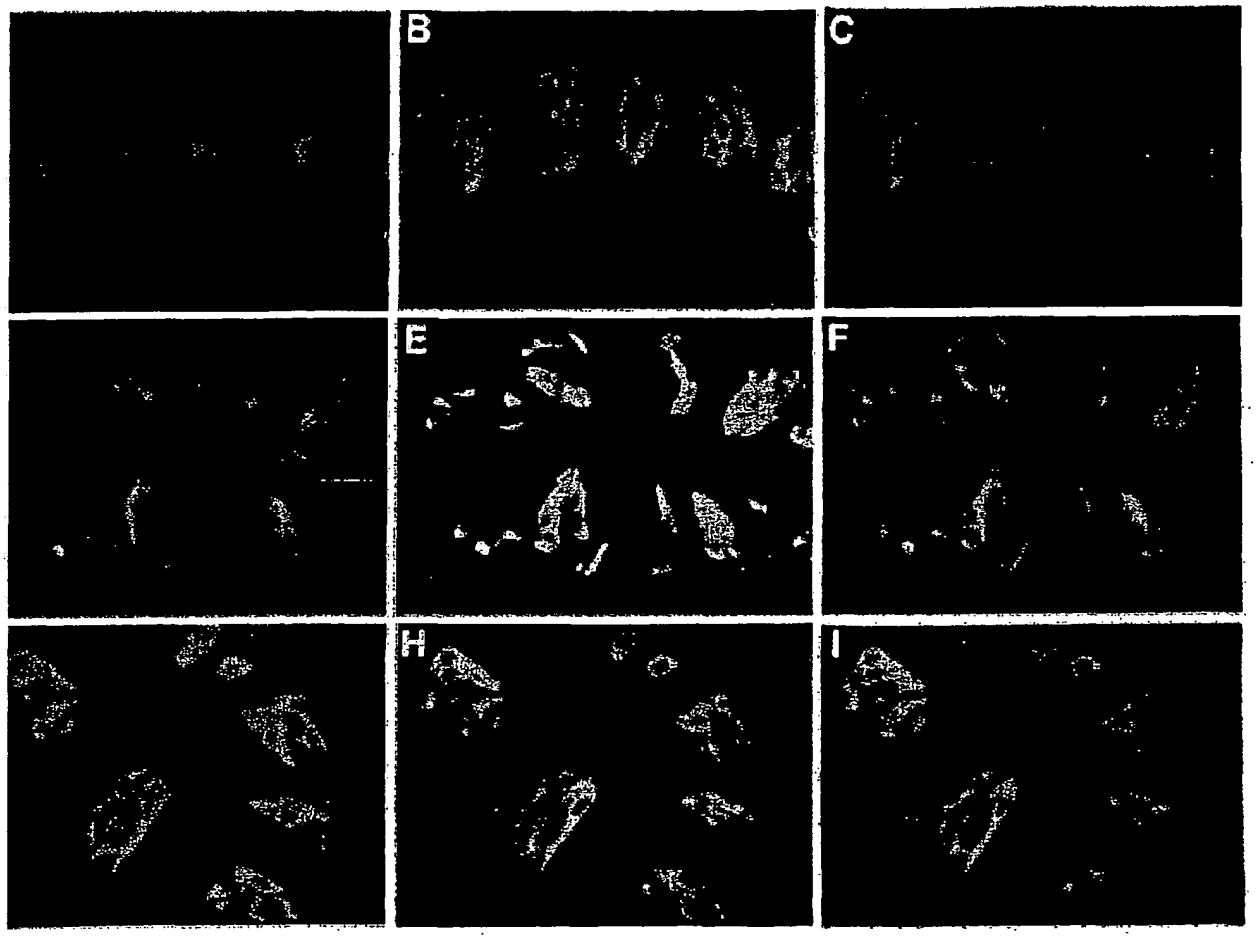

Figure 9

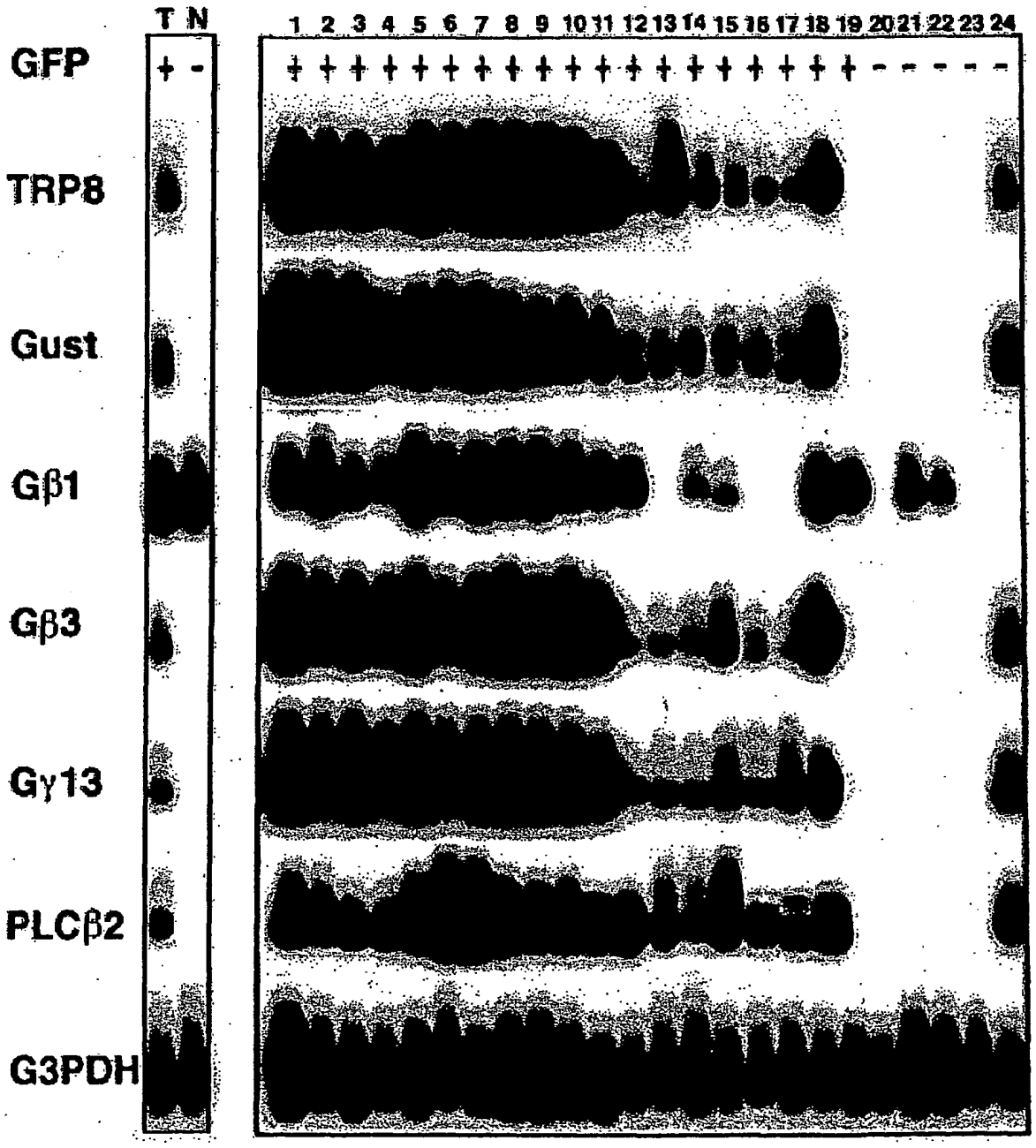
T N
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
GFP
+ -
+ + + + + + + + + + + + + + + + + + + - - - - - -
TRP8
Gust
Gβ1
Gβ3
Gγ13
PLCβ2
G3PDH

Figure 10

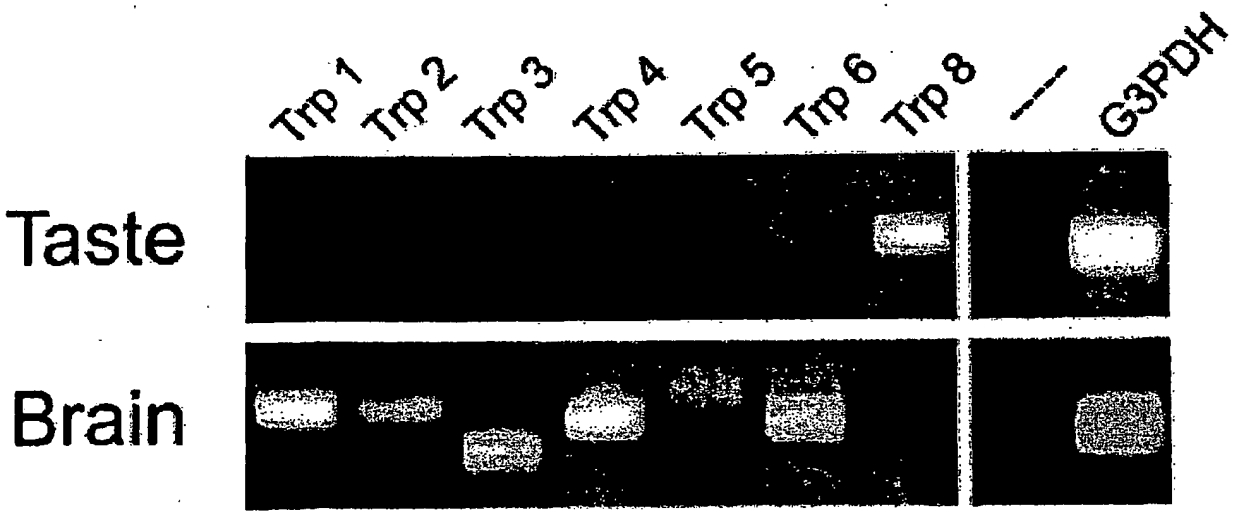

Figure 11

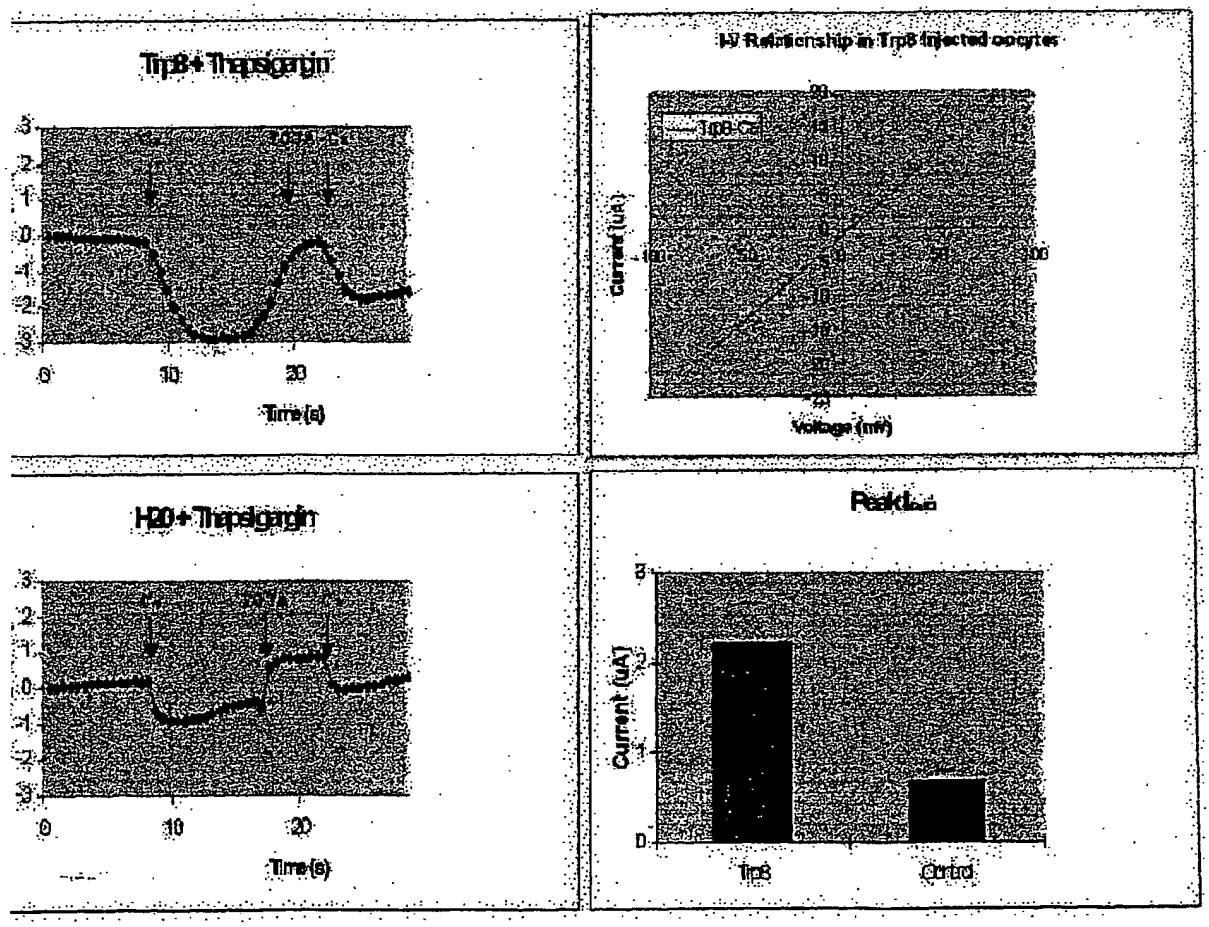
Trp8+ Thapsigargin
Time (s)
I-V Relationship in Trp8 injected oocytes
Trp8-Ca
Current (uA)
Voltage (mV)
H20+ Thapsigargin
Time (s)
Peak I
Current (uA)
Trp8
Control

Figure 12

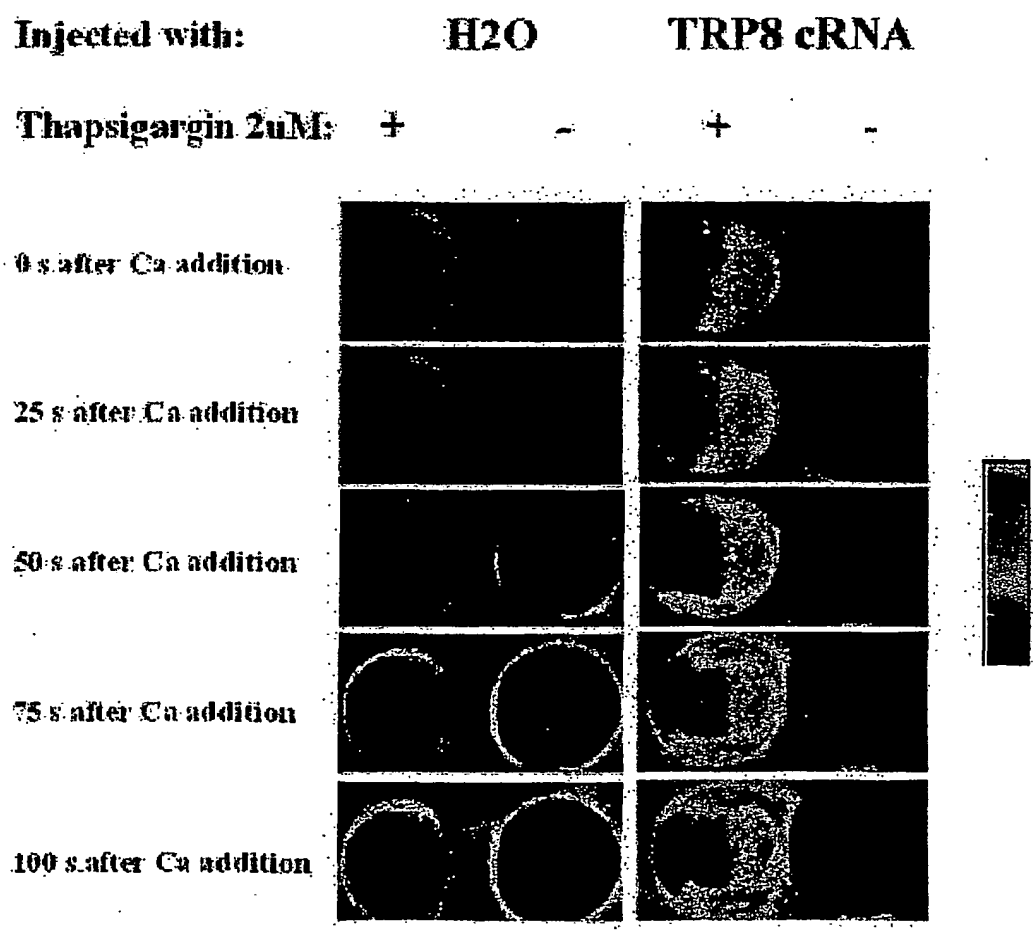
Injected with:
H2O
TRP8 cRNA
Thapsigargin 2uM:
+
-
+
-
0 s after Ca addition
25 s after Ca addition
50 s after Ca addition
75 s after Ca addition
100 s after Ca addition

Figure 13

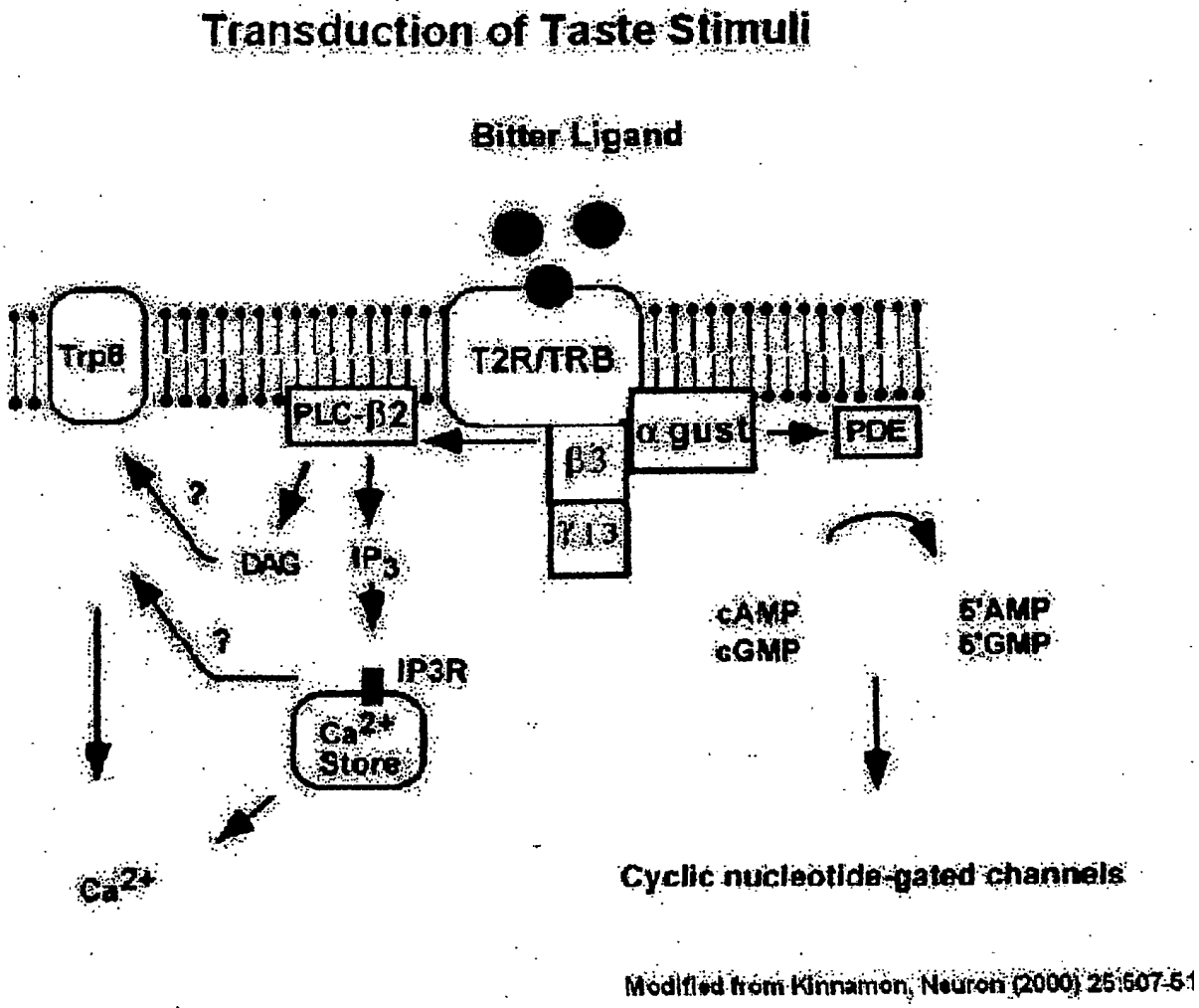
Transduction of Taste Stimuli
Bitter Ligand
Trp8
T2R/TRB
PLC-β2
α gust
PDE
β3
γ13
?
DAG
IP3
?
IP3R
Ca2+ Store
cAMP
cGMP
5'AMP
5'GMP
Ca2+
Cyclic nucleotide-gated channels
Modified from Kinnamon, Neuron (2000) 25:507-51

Figure 14